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QUANTITATIVE INVESTIGATION OF PRECIPITIN REACTIONS OF PROTEINS TREATED WITH PHENYL*ISO*CYANATE AND FORMALDEHYDE.

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IN studies of the chemical basis of serological specificity many methods of preparation of antigens have the disadvantage that, because of the drastic treatment employed to introduce "determinant groups" into the protein molecule, it is possible that other unknown chemical changes or intramolecular rearrangements may occur in the protein molecule and influence the serological specificity. From this standpoint methods which do not involve any violent treatment have a special advantage.

The treatment of proteins with phenyl*iso*cyanate introduced by Hopkins and Wormall (1933) has this advantage. The reaction occurs at 0° C. and at about pH 8·0. Phenyl*iso*cyanate reacts with free amino groups of proteins as follows :

$$R\text{–}NH_2 + O = C = N.C_6H_5 \rightarrow R\text{–}NH - \underset{\underset{O}{\|}}{C} - NH.C_6H_5$$

phenyl*iso*cyanate phenylureidoprotein.

There are indications that NH_2 groups of lysine are mainly concerned (Hopkins and Wormall, 1933, 1934). The purification of the phenyl*iso*cyanate compound of the protein is carried out by adding acetic acid in amount sufficient to cause the maximum precipitation and redissolving the precipitate by the addition of $N/20$ NaOH to a pH not greater than 8·0.

This treatment leads to the acquisition of a new specificity common to all types of proteins treated with phenyl*iso*cyanate. At the same time there is a retention of the original species specificity, since (1) treated proteins give a very strong precipitin reaction with antisera for normal proteins, and (2) antisera

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for treated proteins react much more strongly with the phenyl*iso*cyanate compounds of homologous proteins than with phenyl*iso*cyanate compounds of heterologous proteins, but give no reaction or only a weak one with unaltered homologous proteins. The point of importance is that the introduced substituent is comparatively large and possesses a benzene ring, so that it can function as a " determinant group " from the serological viewpoint. Although the procedure of introduction of this " determinant group " is very mild, even here the possibility of other changes which might influence the specificity cannot be excluded.

Quantitative investigations concerning the alteration of serological specificity of proteins treated with phenyl*iso*cyanate were not carried out by the authors quoted. In the present work a quantitative examination of the precipitin reactions of (1) the antisera produced by unaltered proteins (horse serum globulin, crystalline egg albumin) with these proteins treated with phenyl*iso*cyanate, and (2) the antisera produced by phenylureido-horse serum globulin was carried out.

For comparison some experiments with proteins changed by means of formaldehyde were done. Formaldehyde also blocks the free amino groups of proteins, but without the introduction of any substituent which could be regarded as a " determinant group ".

$$R\text{–}NH_2 + HCHO \rightarrow R\text{–}N = CH_2 + H_2O$$

The experiments with formolized proteins were made only in reaction with anti-normal protein sera. Anti-formolized protein sera, as will be explained in the discussion, do not differ very much from anti-normal protein sera.

METHODS.

Treatment of proteins with formaldehyde.

Several different methods were tried with no appreciable difference in the results of serological experiments. The method finally used was similar to that described by Eagle (1938). To 8 ml. of 4–5 per cent. protein solution (horse serum globulin or crystalline egg albumin) were added 2·6 ml. of $M/4$ phosphate buffer (pH 8·0) or borate buffer (in case of crystalline egg albumin always the latter, because the phosphate buffer caused precipitation) and 0·06 ml. of 40 per cent. formaldehyde ; this mixture was kept for one hour at room temperature and 1 ml. of 10 per cent. $NaHSO_3$ was then added to destroy the excess of formaldehyde.

*Treatment of proteins with phenyl*isocyanate.

The procedure used was similar to that employed by Hopkins and Wormall (1933) ; the only difference was that after adding phenyl*iso*cyanate, mechanical stirring was continued for 5–6 hours at 0° C., the pH being frequently controlled by means of indicators and adjusted to about pH 8·0 by the addition of $N/10$ NaOH. (With crystalline egg albumin borate buffer was used instead of phosphate buffer.)

Methods of estimation.

(1) *Estimations of total nitrogen* were carried out by the micro-Kjeldahl method, and the concentration of the protein solutions calculated from the nitrogen content by using the factor 6·4.

(2) *Free amino-nitrogen* was estimated in a Van Slyke apparatus. In case of horse-serum globulin the free amino-N was reduced from 5·7 per cent. to 2·2 per cent. of the total nitrogen by the treatment with formaldehyde, and to 2·3 per cent. by the treatment with phenyl*iso*cyanate, and in the case of crystalline egg albumin from 6·0 per cent. to 3·2 per cent. with formaldehyde, and to 1·8 per cent. with phenyl*iso*cyanate.

(3) *Optimal proportions in precipitin reactions* were estimated by the method of Dean and Webb (1926): increasing amounts of antigen were mixed with a fixed amount of antiserum, and the proportion in which flocculation appeared first was regarded as optimal.

(4) *Amounts of precipitate* were measured as described by Marrack and Duff (1938): different amounts of the protein solution were added to 1 ml. of antiserum and diluted with sufficient 1 per cent. NaCl to make the final volume up to 3 ml.; all solutions were at 0° C. The mixtures were kept overnight in the refrigerator, the precipitates centrifuged, washed twice with cold 1 per cent. NaCl, and the nitrogen estimated as in (1).

(5) *Qualitative precipitin tests* were made by the ring method.

Immunization of rabbits.

With normal proteins this was carried out by intraperitoneal injections of 4 ml. 2 per cent. antigen solution twice a week for 3 weeks; with phenylureido-horse serum globulin intravenous injections of 1 ml. 1 per cent. antigen solution were made every second day seven times. The animals were bled a week after the last injection. As the antisera to phenylureido-horse serum globulin obtained after this first series of injections were not strong enough, one of the rabbits (105), after a 6 weeks' interval, received a second series of four intravenous injections of 4 ml. of 1 per cent. antigen solution over a period of 2 weeks, and was bled 10 days after the last injection (the corresponding serum was labelled 105*a*).

RESULTS.

Precipitin Reactions of Antisera for Unaltered Proteins with Unaltered Proteins and with Proteins Changed by Treatment with Formaldehyde and with Phenylisocyanate.

The optimal proportions in precipitin reactions of the antiserum for normal horse serum globulin with normal horse serum globulin and with the same globulin treated with formaldehyde and phenyl*iso*cyanate do not reveal any appreciable difference (1·2 mg. of normal globulin, 1·7 mg. of globulin treated with formaldehyde, and 1·7 mg. of globulin treated with phenyl*iso*cyanate to 1 ml. of the antiserum 534.20.12.38; these differences are within the limits of the experimental error).

TABLE I.—*Amounts of Precipitate Formed with Antiserum* 534.24.2.39 *for Normal Whole Horse Serum Globulin.*

Exp. No.	Protein added to 1 ml. antiserum. (mg.)	Protein in ppt. from 1 ml. antiserum. (mg.)	Tests of supernatant fluids with—		
			Antiserum.	Normal horse serum globulin.	Phenylureido-horse serum globulin.
	Normal horse serum globulin.				
1	0·3	1·8	0	+++	+++
2	0·6	3·2	0	±	±
3	1·7	5·0	+	0	0
4	2·2	5·0	++	0	0
5	3·2	4·7	+++	0	0
6	4·5	2·7	+++	0	0
	Phenylureido-horse serum globulin.				
7	0·5	2·1	0	++	++
8	1·0	3·9	0	0	0
9	2·2	5·3	+++	0	0
10	4·4	5·6	+++	0	0

TABLE II.—*Amounts of Precipitate Formed with Antiserum* 534.20.12.38 *for Normal Whole Horse Serum Globulin.*

Exp. No.	Protein added to 1 ml. antiserum. (mg.)	Protein in ppt. from 1 ml. antiserum. (mg.)	Tests of supernatant fluids with—		
			Antiserum.	Normal horse serum globulin.	Formolized horse serum globulin.
	Normal horse serum globulin.				
1	0·6	3·1	±	+++	+++
2	1·2	4·3	++	0	0
3	1·7	4·7	+++	0	0
4	2·4	4·8	+++	0	0
5	3·0	4·9	+++	0	0
6	3·4	4·7	+++	0	0
7	4·8	2·6	+++	0	0
	Formolized horse serum globulin.				
8	0·6	2·8	0	+++	+++
9	0·8	3·6	±	++	++
10	1·2	4·5	+	0	0
11	1·7	5·4	++	0	0
12	2·4	5·9	+++	0	0
13	3·4	6·2	+++	0	0
14	4·8	6·0	+++	0	0

Estimations of the amounts of precipitate, however, show some differences. The results of these estimations are shown in Tables I and II and by Figs. 1 and 2. In the antigen excess zone the amounts of precipitate formed with changed globulins are greater than the corresponding amounts formed with the unaltered globulin. The reverse seems to be the case in the zone of antibody excess. Thus the curves for the changed globulins reach higher levels than those for the unaltered globulin. The most important difference is that

FIG. 1.

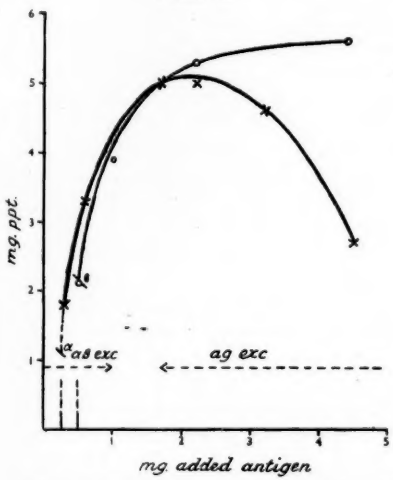

FIG. 2.

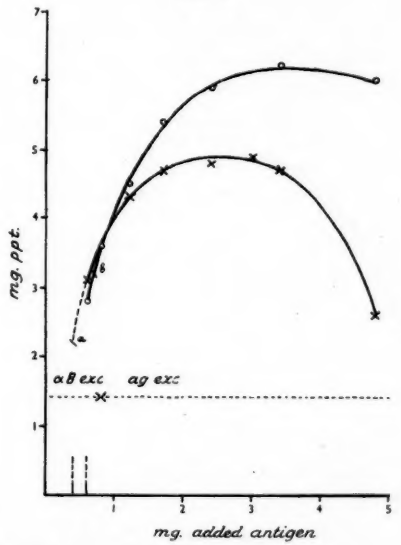

FIG. 1.—Antiserum to normal horse serum globulin (534.24.2.39). o = phenylureido-horse serum globulin. × = normal horse serum globulin. *ab. exc.* = antibody excess. *ag. exc.* = antigen excess.

FIG. 2.—Antiserum to normal horse serum globulin (534.20.12.38). o = formolized horse serum globulin. × = normal horse serum globulin. *ab. exc.* = antibody excess. *ag. exc.* = antigen excess.

in the zone of greater excess of antigen the fall of the amount of precipitate (due to the formation of soluble antigen-antibody compounds) is much more marked with normal globulin than with changed globulins. The ability of changed globulins to form soluble compounds with antibody is much less than that of unaltered globulin, but this may be due simply to the change of physical properties of the globulin molecule caused by the treatment with formaldehyde or phenyl*iso*cyanate. This would account for the results described above.

Marrack and Smith (1931) found that the stronger the serological reaction of antiserum, the greater the amount of precipitate formed from a given amount of serum; moreover they found that the ratio of antigen to total protein in the precipitate (which increases with increasing amount of added

antigen) rises with increasing strength of antiserum. In our case the amounts of precipitate obtainable from a given amount of the antiserum are higher with changed globulins than with normal globulin, even though it was the latter which gave rise to the production of the antisera. But the ratio of antigen found in the precipitate to the total amount of precipitate (as it was shown by means of two experiments described below) is higher with unaltered globulin than with changed globulins under the same conditions. It seems therefore that the affinity of the unaltered globulin for the antibody is stronger than that of changed globulins.

Estimation of the amount of antigen in supernatants.

As was shown by Heidelberger and Kendall (1935), the estimation of very small amounts of antigen in supernatants in the limited range of antigen excess before the beginning of the inhibition zone can be most simply carried out by adding the supernatant to a measured amount of the antiserum used in the determinations of total precipitate. The amount of precipitate so formed can be estimated and the corresponding amount of antigen read off from the total precipitate curve. In the two following experiments the amounts of antigen in several supernatants of Tables I and II were estimated. The ratios of antigen bound in the precipitate to the total amount of precipitate were calculated.

Experiment 1.—Supernatants No. 4 and No. 9 (Table I) were mixed with 1 ml. of the antiserum 534.24.2.39. The amounts of precipitate were 1·1 mg. (No. 4) and 2·1 mg. (No. 9), which correspond respectively to about 0·25 mg. (Fig. 1, point *a*) and 0·5 mg. (point *b*) of antigen in the supernatants. The amount of antigen bound in the precipitate No. 4 (*ca.* 2·2 mg.–0·25 mg. = 1·95 mg.) is thus higher than in the precipitate No. 9 (*ca.* 2·2 mg.–0·5 mg. = 1·7 mg.), and therefore the ratio of antigen to total precipitate is higher in the precipitate No. 4 (1·95 : 5·0 = 0·39) than in No. 9 (1·7 : 5·3 = 0·32).

Experiment 2.—Supernatants No. 3 and No. 11 (Table II) were mixed with 1 ml. of the antiserum (534.20.12.38). The amounts of precipitate were 2·2 mg. (No. 3) and 3·2 mg. (No. 11), which correspond respectively to about 0·4 mg. (Fig. 2, point *a*) and 0·7 mg. (point *b*) of antigen in supernatants. The amount of antigen bound in the precipitate No. 3 (*ca.* 1·7 mg.–0·4 mg. = 1·3 mg.) is thus higher than in the precipitate No. 11 (*ca.* 1·7 mg.–0·7 mg. = 1·0 mg.), and therefore the ratio of antigen to total precipitate is higher in the precipitate No. 3 (1·3 : 4·7 = 0·27) than in the precipitate No. 11 (1·0 : 5·4 = 0·18).

(Both experiments were carried out in the zone of only slight excess of antigen before the beginning of the inhibition zone. Therefore the amounts of antibody in the supernatants, as soluble antigen-antibody compounds, could not yet be great enough to cause great errors in the calculations.)

Experiments with crystalline egg albumin.

Experiments analogous to those described above were carried out using the antiserum for unaltered crystalline egg albumin and unaltered crystalline egg albumin and egg albumin treated with formaldehyde and with phenyl*iso*-cyanate as antigens.

The optimal proportions in the precipitin reaction of unaltered egg albumin and of formolized egg albumin were the same (0·6 mg. to 1 ml. of antiserum 79.27.1.39). But there was a difference between unaltered egg albumin and egg albumin treated with phenyl*iso*cyanate which was great enough to be outside the limits of the experimental error : 1·2 mg. of unaltered egg albumin, and 3·8 mg. of egg albumin treated with phenyl*iso*cyanate to 1 ml. of the antiserum (81.27.1.39).

TABLE III.—*Amounts of Precipitate Formed with Antiserum* 79.27.1.39 *for Normal Crystalline Egg Albumin.*

Exp. No.	Protein added to 1 ml. antiserum. (mg.)	Protein in ppt. from 1 ml. antiserum. (mg.)	Tests of supernatant fluids with— Antiserum.	Normal egg albumin.	Formolized egg albumin.
	Normal crystalline egg albumin.				
1	0·3	3·6	±	++	Trace
2	0·6	5·3	++	0	0
3	1·2	4·3	+++	0	0
	Formolized crystalline egg albumin.				
4	0·3	3·2	±	+++	++
5	0·6	3·7	++	0	0
6	1·2	3·0	+++	0	0

TABLE IV.—*Amounts of Precipitate Formed with Antiserum* 81.27.1.39 *for Normal Crystalline Egg Albumin.*

Exp. No.	Protein added to 1 ml. antiserum. (mg.)	Protein in ppt. from 1 ml. antiserum. (mg.)	Tests of supernatant fluids with— Antiserum.	Normal egg albumin.	Phenylureido-egg albumin.
	Normal crystalline egg albumin.				
1	0·25	5·2	0	0	0
2	0·5	5·3	+++	0	0
3	1·0	1·7	+++	0	0
4	2·0	—			
	Phenylureido egg albumin.				
5	0·5	2·3	0	+++	+++
6	1·0	3·0	0	++	0
7	2·0	4·8	+++	0	0
8	4·0	1·6	+++	0	0

The results of the estimations of the amounts of precipitate are recorded in Tables III and IV. The amounts of precipitate obtained with formolized egg albumin were smaller than the corresponding amounts formed with unaltered egg albumin (Table III). The highest points of both curves correspond to 0·6 mg. of antigen added to 1 ml. of the antiserum.

Phenylureido-egg albumin reacted in a different manner. Here also the amounts of precipitate were smaller than those formed with unaltered egg albumin, but the maximum was only reached when the amount of added antigen was about four times as great as the amount of added unaltered egg albumin at its maximum (Table IV). This is consistent with the fact that the optimal proportions show in this case nearly the same difference.

The Precipitin Reactions of Antisera to Phenylureido-Horse Serum Globulin.

Table V records the results of qualitative tests of four antisera produced by injecting rabbits with the solution of phenylureido-horse serum globulin. One of these sera (104) gave a weak reaction only with phenylureido-horse serum globulin. The stronger sera (105, 105*a*) reacted with proteins of other species treated with phenyl*iso*cyanate, but much less strongly than with phenylureido-horse serum globulin. With unaltered horse globulin these sera gave only a very weak reaction. These results confirm those of Hopkins and Wormall (1933).

TABLE V.—*Qualitative Tests (Ring) with Antisera for Phenylureido-Horse Serum Globulin.*

Antigens.	Antisera.			
	102.	104.	105.	105*a*.
Phenylureido-horse serum globulin	++	+	+++	++++
Horse serum globulin	—	—	Trace	+
Phenylureido-rabbit serum protein	Trace	—	++	+++
Phenylureido-egg albumin	—	—	+	+++
Egg albumin	—	—	—	—
Phenylureido-man serum globulin	Trace	—	++	
Man serum globulin	—	—	—	

The results of estimations of the amounts of precipitate obtained with the antiserum 105 are given in Table VI. All the amounts of precipitate were very small. They were greater with phenylureido-horse serum globulin than with phenylureido proteins of other species. The precipitate formed with unaltered horse serum globulin was so small that its quantitative estimation was impossible.

TABLE VI.—*Amounts of Precipitate Formed with Antiserum* 105 *for Phenylureido-Horse Serum Globulin.*

Exp. No.	Protein added to 1 ml. antiserum. (mg.)	Protein in ppt. from 1 ml. antiserum. (mg.)	Tests of supernatant fluids with— Antiserum.	Phenylureido-horse globulin.	Horse globulin.	Phenylureido-egg albumin.	Phenylureido-rabbit protein.	Phenylureido-man serum globulin.
	Phenylureido-horse globulin.							
1	0·3	0·5	±	0	0			
2	0·7	0·6	++	0	0			
3	1·6	0·7	++	0	0			
4	3·3	0·6	++	0	0			
	Phenylureido-egg albumin.							
5	2·2	Trace						
6	3·3	0·3	+	+	±	0	0	
7	4·4	0·3	++	±	0	0	0	
	Phenylureido-rabbit protein.							
8	1·1	0·2	±	++	±	0	0	
9	2·2	0·3	++	+	0	0	0	
10	3·3	0·3	++	+	0	0	0	
11	4·4	0·3	++	±	0	0	0	
	Phenylureido-man serum globulin.							
12	0·75	0·2	+	+				0
13	1·5	0·3	++	+				0
14	2·25	0·3	++	+				0
15	3·0	0·3	++	±				0

The amounts of precipitate formed by the antiserum 105*a* (see p. 10), resulting from immunization of the rabbit 105 with a second series of injections, are given in Table VII. The reaction with phenylureido-horse serum globulin was quite a strong one, giving a curve very similar to those of sera for normal horse globulin reacting with treated horse serum globulin (Figs. 1 and 2). However, with phenylureido-proteins of other species (egg albumin, rabbit serum protein) the amounts of precipitate were much less than with phenylureido-horse globulin. The amounts of precipitate formed with untreated horse globulin were very small.

TABLE VII.—*Amounts of Precipitate Formed by Antiserum 105a for Phenylureido-Horse Serum Globulin.*

Exp. No.	Protein added to 1 ml. antiserum. (mg.)	Protein in ppt. from 1 ml. antiserum. (mg.)	Tests of supernatant fluids with— Antiserum.	Phenylureido-horse globulin.	Horse globulin.	Phenylureido-rabbit protein.	Phenylureido-egg albumin.
	Phenylureido-horse globulin.						
1	0·5	1·6	0	+++	+	0	0
2	1·0	3·4	0	±	0	0	0
3	1·75	4·3	±	0	0		
4	3·5	5·2	++	0	0		
5	5·3	5·6	+++	0	0		
6	7·0	5·65	+++	0	0		
	Normal horse globulin.						
7	2·5	0·3	++	+++	0	0	
8	5·0	0·3	++	+++	0	0	
	Phenylureido rabbit protein.						
9	0·5	0·5	+	+++	0	0	
10	1·25	0·7	++	+++	0	0	
11	2·5	1·1	+++	+++	0	0	
12	5·0	1·2	+++	+++	0	0	
	Phenylureido-egg albumin.						
13	1·25	0·4	+	+++	0		0
14	2·5	0·6	++	+++	0		0
15	5·0	0·7	++	+++	0		0

There is, therefore a very great quantitative difference between the reaction of phenylureido-horse serum globulin and the reaction of other tested antigens. The difference is also accentuated by the inability of these latter to exhaust the antiserum and cause the lack of reaction with phenylureido-horse globulin (the tests of the respective supernatants with phenylureido-horse globulin were all strongly positive—Table VII).

The observation of Hopkins and Wormall (1933) that phenylureido-horse globulin (which reacts with anti-normal globulin sera nearly as strongly as does the normal horse globulin) when injected into a rabbit gives rise to the production of an antiserum reacting strongly only with phenylureido-horse globulin, but not with the normal horse globulin, has been confirmed by the present quantitative work.

DISCUSSION.

The fact that the treatment of proteins with phenyl*iso*cyanate or formaldehyde only slightly reduces their affinity towards antibodies for corresponding normal proteins and that treated proteins bind all the antibody, so that supernatants give no reactions with normal protein, seems to show that the species determinant groups are still strongly active, and that NH_2 groups of the antigenic protein are not involved in combination with the antibody. The greater effect of phenyl*iso*cyanate compared with that of formaldehyde (which is specially evident in case of egg albumin, *cf.* Tables III and IV) may be the result of the introduction of comparatively large substituent groups which affect the approach of antigen reacting groups to antibody molecules.

The peculiar phenomenon that globulins changed by means of phenyl*iso*cyanate as well as by formaldehyde in the excess antigen zone form more precipitate with antisera to normal globulin than the normal globulin does (in spite of somewhat reduced affinity towards the antibody) may be caused by a change in physical properties of the treated antigen molecules, so that their ability to form soluble antigen-antibody compounds diminishes ; there may be a special kind of antibody, which forms no insoluble compound with the normal globulin, but does so with the changed globulins.

Results of immunization of rabbits with phenylureido-globulin show that species determinant groups, which *in vitro* are fully active with anti-normal globulin sera, are dominated *in vivo* by groupings produced by phenyl*iso*cyanate and only very slightly stimulate the animal organism to production of antibodies, while these dominating groupings cause the production of great amount of antibodies specific for them. (A one-sided cross-reaction described by Heidelberger and Kendall (1934) is an instance in which the reaction of species determinant groups with antisera to normal protein was masked by artificially introduced azo-groupings, while *in vivo* these groups became reactive and caused the formation of corresponding antibody.)

The majority of the antibody molecules combine with antigen molecules which carry both the species specific and phenylureido-groups and, therefore, react only with phenylureido-horse globulin, and not with phenylureido proteins of other species nor with unchanged horse globulin (*cf.* Haurowitz, 1937). The antibodies for phenylureido groups, which react with phenylureido proteins of other species (including rabbit protein), appear in much smaller amount.

Species determinant groups of formolized proteins, strongly active *in vitro* with corresponding anti-normal protein sera, are also fully active *in vivo* and stimulate animal organisms to produce antibodies reacting strongly with corresponding normal proteins (Landsteiner and Lampl, 1917 ; Horsfall, 1934 ; Jacobs and Sommers, 1939). The acquisition of a new specificity characteristic for all types of formolized proteins, shown by Horsfall (1934) contrary to earlier investigators (Landsteiner and Lampl, 1917 ; Eisler and Löwenstein, 1912), was not confirmed recently by Jacobs and Sommers (1939), who used all the methods of formolization described by earlier authors, and could hardly demonstrate it even within close limits of zoological relationship. Thus this new formaldehyde specificity seems to be very doubtful.

The difference between the influence of phenyl*iso*cyanate and formaldehyde on antigenical properties of proteins is understandable since in the first case a comparatively large new group containing the benzene ring is introduced, while in the second case, apart from the blockage of free NH_2 groups, nothing which could be regarded as a new " determinant group " is introduced or created.

SUMMARY.

Quantitative experiments have shown that—

(1) Horse serum globulin and crystalline egg albumin treated with phenyl-*iso*cyanate and with formaldehyde give a strong precipitin reaction with rabbit antisera produced by corresponding unchanged proteins, but there are certain quantitative differences between their reaction and the reaction of unchanged proteins.

The treatment with phenyl*iso*cyanate or with formaldehyde causes some reduction of the affinity towards antibodies for corresponding normal proteins. Some quantitative changes of precipitin reaction presumably are due to changes of physical properties of the antigen molecules.

(2) Immunization of rabbits with phenylureido-horse serum globulin gave rise to antisera reacting strongly with phenylureido-horse globulin, much less strongly with phenylureido proteins of other species and only very slightly with normal horse serum globulin.

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CHEMOTHERAPEUTIC EXPERIMENTS ON PLEURO-PNEUMONIA-LIKE ORGANISMS IN RODENTS.

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IN a previous communication (Findlay, Mackenzie, MacCallum and Klieneberger, 1939) brief reference was made to the action of gold and arsenic in preventing the development of polyarthritis in the rat, as the result of inoculating cultures of a pleuropneumonia-like organism (L7) into the pad. Collier (1939), who, however, was unable to isolate a causal organism from a similar infective polyarthritis in the rat, had also shown that two gold preparations, solganal B and aurodetoxin, had the power of preventing the development of arthritic changes in the rat. Chemotherapeutic experiments are now described on five pleuropneumonia-like organisms isolated from rodents commonly used in the laboratory. Two of these organisms, L5 and L6, were isolated from mouse brains (Findlay, Klieneberger, MacCallum and Mackenzie, 1938, 1939), two from rats, L4 (Klieneberger, 1939*a*) and L7 (Findlay, Mackenzie, MacCallum and Klieneberger, 1939) and one from guinea-pigs (Klieneberger, 1939*b*). Of the organisms isolated from mouse brains L5 produces rolling and choreic-like symptoms on intracerebral injection of mice (rolling disease), associated with a high mortality. On inoculation into the pad of the foot of mice the same organism produces arthritic changes in from 30 to 50 per cent. of animals. L6, which is slightly different in its growth characteristics and serologically distinct from L5, also produces arthritis in approximately the same percentage of mice. These two organisms are not pathogenic for rats or guinea-pigs. L4 is a pleuropneumonia-like organism, originally obtained by Klieneberger (1939*a*) from abscesses in the rat, and described by Woglom and Warren (1938*a*, 1938*b*, 1939) as a "pyogenic virus". This organism invariably causes arthritis in rats and mice when injected into the pad. It is closely similar serologically, though somewhat more virulent than L7, which was obtained from a spontaneous arthritis of the rat. L7 causes an arthritis in rats and also in mice when injected into the pad. All the four organisms, L4, L5, L6 and L7, are unassociated with anything resembling *Streptobacillus moniliformis*. The guinea-pig organism was obtained by Klieneberger (1939*b*) from abscesses in the cervical region of guinea-pigs. This pleuropneumonia-like organism is associated with an organism resembling *Streptobacillus moniliformis*. It has also been obtained from guinea-pigs by Wilson Smith (Klieneberger, 1939*b*), while we have isolated it from abscesses in our own laboratory stock guinea-pigs and in a guinea-pig obtained in Essex by Dr. P. Martin of the Hospital for Tropical Diseases. When injected intradermally into the pad of mice this organism produces an arthritis in all mice.

EXPERIMENTAL TECHNIQUE.

The following compounds were tested for chemotherapeutic action: sulphanilamide, sulphapyridine, sodium salicylate, neoarsphenamine, acetarsol, tryparsamide, lithium antimonyl chloride, solganal B, neosolganal, myocrysin, lopion and parmanil. Blood broth cultures of L4 and L7 were injected into the pads of mice and rats in doses of 0·1 c.c. L4, L5 and L6 were inoculated intracerebrally into mice in doses of 0·03 c.c., while the guinea-pig organism was injected into the pads of mice in doses of 0·1 c.c. Sulphanilamide and sulphapyridine were given either subcutaneously or *per os* suspended in gum acacia; the other compounds were injected subcutaneously into the groin. Except when stated the first dose of the drug was given immediately after injection of the organism; subsequent doses were given at intervals of 24 hours.

RESULTS OF CHEMOTHERAPEUTIC EXPERIMENTS.

Sulphonamide Compounds.

Sulphanilamide.

Sulphanilamide suspended in gum acacia was given *per os* immediately after infection, and thereafter for four days in doses of 2·5 g. per kilogram of body-weight to batches of 20 mice injected intracerebrally with L4, L5 and L6 respectively; batches of 20 mice were injected as controls with the same organisms, but received no treatment. The death-rate among the treated mice was of the same order as among the controls. Similar results were obtained when sulphanilamide was injected subcutaneously in oil in doses of 3 g. per kilogram of body-weight.

Sulphapyridine.

Results similar to the above were obtained when sulphapyridine was given either *per os* or subcutaneously.

Arsenical Compounds.

Neoarsphenamine.

Neoarsphenamine was given to mice in doses of 2·5 mg. per 20 g. of body-weight subcutaneously immediately after intracerebral injection of L4, L5 and L6 and thereafter at intervals of 24 hours, four injections in all being given. The results are shown in Table I. The most striking results were obtained with L5, the agent of rolling disease; 43·75 per cent. of the treated animals survived as against 20·8 per cent. of the untreated controls.

TABLE I.—*Neoarsphenamine and Pleuropneumonia-like Organisms in Mice.*

Organism.	Treated mice.			Percentage survived.	Control mice.			Percentage Survived.
	Number.	Sick.	Died.		Number.	Sick.	Died.	
L4	48	36	36	25·0	48	44	40	16·6
L5	48	27	27	43·75	48	44	38	20·8
L6	48	20	12	75·0	48	32	20	58·3

A series of 24 mice injected in the pad with L7 were also treated with the same doses of neoarsphenamine: 18 developed arthritis, while of the 24 controls, all developed arthritic lesions.

Acetarsol.

Acetarsol, acetylaminohydroxyphenylarsonic acid, was injected subcutaneously in batches of 12 mice in doses of 25 mg. per 20 g. of body-weight immediately after intracerebral injection of L4, L5 and L6 and thereafter at intervals of 24 hours. Four similar injections were given. The results are shown in Table II; the best results were again obtained with L5.

TABLE II.—*Acetylaminohydroxyphenylarsonic Acid (Acetarsol) and Pleuropneumonia-like Organisms in Mice.*

Organism.	Treated mice.			Control mice.		
	Number.	Sick.	Died.	Number.	Sick.	Died.
L4	12	12	10	12	12	11
L5	12	8	4	12	11	9
L6	12	8	6	12	12	10

TABLE III.—*Tryparsamide and Pleuropneumonia-like Organisms in Mice.*

Organism.	Treated mice.			Percentage survived.	Control mice.			Percentage survived.
	Number.	Sick.	Died.		number.	Sick.	Died.	
L4	36	35	32	11·1	36	35	31	13·9
L5	36	23	22	38·8	36	35	25	35·5
L6	36	23	18	50·0	36	30	24	33·3

Tryparsamide.

Tryparsamide was injected subcutaneously in doses of 20 mg. per 20 g. of body-weight in batches of 36 mice immediately after intracerebral injection of L4, L5 and L6. The results in the treatment of L4 and L5 were unsatisfactory, but with L6 a slight preventive action was demonstrable. The results are shown in Table III.

Sodium Salicylate.

In view of the reputed action of sodium salicylate in human rheumatic infections, the effects of this compound were tested on L7 injected into the pads of mice and L4, L5, L6 and L7 injected intracerebrally in mice. The sodium salicylate was injected subcutaneously in the first experiment in doses of 10 mg. per 20 g. of body-weight, and in the second experiment of 20 mg. per 20 g. of body-weight, immediately after injection of the cultures and thereafter on three occasions at intervals of 24 hours. No difference was found in either of the experiments in the number of treated and control mice, either in the development of arthritis due to L7 or in the development of cerebral symptoms following intracerebral injection of L4, L5, L6 and L7.

Antimony Compounds.

In view of the slight activity of arsenic in preventing symptoms, an attempt was made to determine whether antimony compounds had a similar action. Lithium antimonyl tartrate, in a solution of which 1 c.c. contained 0·0036 g. Sb, was injected subcutaneously in doses of 0·05 c.c. per 20 g. of body-weight into mice inoculated into the pad with L7 and the guinea-pig organism. Both the treated and untreated animals developed symptoms in equal numbers in approximately the same period after infection. Under the experimental conditions antimony was thus without action.

Gold Salts.

The effect of sodium aurothioglucose, solganal B, on the development of symptoms following the intracerebral injection of L4, L5 and L6 in mice was first investigated. Solganal B is said to contain 50 per cent. of gold. The drug was given subcutaneously in a dose of 10 mg. per 20 g. of body-weight immediately after intracerebral injection of the organisms; thereafter four similar injections were given at intervals of 24 hours. The results are seen

TABLE IV.—*Solganal B and Pleuropneumonia-like Organisms in Mice.*

Organism.	Treated mice.			Percentage survived.	Control mice.			Percentage survived.
	Number.	Sick.	Died.		Number.	Sick.	Died.	
L4	24	4	4	83·3	24	22	19	20·8
L5	24	4	4	83·3	24	24	22	8·3
L6	24	2	2	91·6	24	19	13	45·8

in Table IV. In the case of all three organisms a much higher percentage of animals treated with solganal B survived as compared with untreated controls. In Table V are shown the results of treating rats, injected into the pad with L7, with solganal B and myocrysin. The compounds were injected subcutaneously immediately after inoculation of 0·2 c.c. of a culture of L7 into the right hind pads of rats; thereafter three similar injections were given at intervals of 24 hours. Of 12 control rats, 10 developed arthritis.

TABLE V.—*The Effect of Solganal B and Myocrysin on the Development of Arthritis in Rats Inoculated with L7.*

Compound.	Dose mg. per 100 g. body-weight.	Treated rats.		Control rats.	
		Number.	Sick.	Number.	Sick.
Solganal B	10	6	0	6	5
Myocrysin	2·5	6	0	6	5

The protective action of organic gold compounds on the development of arthritis in mice following the injection of L7 into the pads is shown in Table VI.

The drugs were injected subcutaneously in the right groin immediately after injection of 0·1 c.c. of a blood broth culture into the pad of the right

foot. Thereafter three similar injections were given at intervals of 24 hours. All the compounds studied, solganal B, myocrysin, lopion, parmanil and neosolganal, showed a protective action against the development of arthritis due to L7. Myocrysin is sodium aurothiomalate with a gold content of 50 per cent.; lopion is sodium aurothiosinamine benzoic acid with a gold content of 43·5 per cent.; parmanil is described as a methyl glucamide of aurothiodiglycollic acid with a gold content of 50 per cent.; while neosolganal is said to be a " calcium gold keratinate " with a gold content of 14 per cent.

TABLE VI.—*Organic Gold Compounds and the Development of Arthritis in Mice Inoculated with Cultures of L7.*

The drugs were injected subcutaneously in the right groin immediately after injection of 0·1 c.c. of culture into the pad of the right foot and thereafter 3 similar injections were given at intervals of 24 hours.

Compound.	Dose mg. per 20 g. of body-weight.	Treated mice.		Control mice.	
		Number developing arthritis.	Number protected.	Number developing arthritis.	Number protected.
Solganal B	10·0	0	24	24	0
Myocrysin	20·0	0	24	24	0
	2·5	16	8	24	0
Lopion	2·5	0	24	24	0
Parmanil	2·5	0	24	24	0
Neosolganal	10·0	8	16	21	3

To test the effect of solganal B on the guinea-pig organism, cultures containing both the pleuropneumonia-like organism and *Streptobacillus moniliformis* were inoculated intradermally together into the pads of the hind feet of mice, each mouse receiving 0·1 c.c. of a 24-hour blood-broth culture. Immediately after the injection of the culture each mouse was given 10 mg. of solganal B per 20 g. of body-weight subcutaneously, and thereafter the same dose was repeated on three occasions at intervals of 24 hours. Each mouse thus received 40 mg. of solganal B per 20 g. of body-weight. The results are shown in Table VII. The 12 control mice all exhibited a progressive swelling of the foot and tibio-tarsal joint and in one animal the arthritis appeared in other joints. Of the treated animals 3 of the 12 showed slight swelling 48 hours after inoculation, but instead of progressing the swelling of the feet gradually disappeared and 8 days after inoculation had completely disappeared.

To determine whether the solganal B had completely destroyed both the pleuropneumonia-like organism and *Streptobacillus moniliformis* two of the treated mice were killed 14 days after inoculation into the foot. Cultures of the inoculated hind foot, blood, liver and spleen remained sterile, while the same tissues inoculated into the pads of normal mice failed to cause any swelling. It is, therefore, concluded that the pleuropneumonia-like organism

and *Streptobacillus moniliformis* were both killed by the gold salt. Three weeks after inoculation the remaining treated mice and six normal controls were inoculated into the pad with cultures of the guinea-pig organism. The treated mice exhibited a progressive swelling of the foot and arthritis after the same interval as the controls and *Streptobacillus moniliformis* and the pleuropneumonia-like organism were obtained from the affected joints. There was thus no evidence that the treated mice had acquired an immunity against the guinea-pig organism.

TABLE VII.—*Development of Arthritis after Inoculation into Pad.*

The effect of solganal B on the development of arthritis in mice inoculated into the pad with the guinea-pig organism (*Streptobacillus moniliformis* + pleuropneumonia-like organism).

Number of animal.	Treated mice. Days after infection.					Control mice. Days after infection.				
	2.	3.	4.	8.	12.	2.	3.	4.	8.	12.
1	+	+	+	−	−	+	++	+++	++++	++++
2	+	+	±	−	−	+	++	+++	+++	+++
3	+	±	−	−	−	+	+	++	+++	+++
4	−	−	−	−	−	+	+	++	+++	+++
5	−	−	−	−	−	++	++	++	+++	+++
6	−	−	−	−	−	+	+	+++	+++	+++
7	−	−	−	−	−	+	+	++	+++	+++
8	−	−	−	−	−	++	++	+++	+++	+++
9	−	−	−	−	−	−	+	++	+++	+++
10	−	−	−	−	−	−	+	++	+++	+++
11	−	−	−	−	−	−	+	+	++	+++
12	−	−	−	−	−	−	+	+	++	+++

− = No swelling of foot or arthritis of tibiotarsal joint.
± = Very slight swelling of foot.
\+ = Swelling of foot.
++ = Swelling of foot and swelling of tibiotarsal joint.
+++ = Marked swelling of foot and swelling of tibiotarsal joint.
++++ = Polyarthritis.

Similar experiments were carried out in the case of the other pleuropneumonia-like organisms. The brains of 2 mice which had failed to develop symptoms when treated 14 days previously with solganal B after intracerebral injection of L5 were removed. One-half of the brains was inoculated intracerebrally into normal mice, while the other half was cultivated in blood broth. No pleuropneumonia-like organisms developed in culture, nor did the inoculated mice exhibit encephalitic symptoms. The mice were subsequently not immune to intracerebral injection of L5. In the same way mice, which as the result of treatment with myocrysin had failed to develop symptoms after intracerebral injection of L4 and L6, were not immune when injected

3 weeks later with the same organisms. Mice which had failed to develop arthritis due to L7, following gold treatment, were also not immune when re-injected with the same organism in the pad of the opposite foot.

In the experiments already described the compounds to be tested for therapeutic action were injected immediately after the inoculation of the pleuropneumonia-like organisms. In the following experiment the mice after injection of cultures of L4 and L7 into the pads were treated with solganal B, (1) immediately after inoculation of the cultures and 24 and 48 hours after inoculation; (2) 24, 48 and 72 hours after inoculation; (3) 48, 72 and 96 hours after inoculation; (4) 72, 96 and 120 hours after inoculation.

From the results in Table VIII it will be seen that solganal B in a dose of 10 mg. per 20 g. of body-weight given immediately after infection prevented the development of arthritis in all the mice injected with L4 and in at least half those injected with L7. When treatment was delayed for 24 hours the preventive action was less in evidence, but was more marked with L4 than with L7. 48 hours after injection the majority of the untreated mice had developed arthritis after infection, both with L4 and L7. In the mice infected with L4 and L7, there was evidence of curative action while some slowing up of the virulence of the infection was also noticeable, for of the untreated control mice inoculated with L7, 6 of the 24 were dead 96 hours after infection, while none of the mice receiving solganal B succumbed. In other experiments in which solganal B was not given till 4 or 5 days after infection with L7, there also appeared to be some slowing up of the infective process, but since the swelling and arthritis are self-limiting and tend to disappear eventually in the absence of any treatment, it is not easy to judge how far solganal B affects the process once it is fully developed.

TABLE VIII.—*Development of Arthritis in Mice after Inoculation of L4 and L7 into the Pad.*

Treatment begun immediately after injection (mice 1–24); 24 hours after injection (mice 25–48); 48 hours after injection (mice 49–72); 72 hours after injection (mice 73–96).

Number of mice.	Treated mice developing arthritis. Days after infection.					Control mice developing arthritis. Days after infection.				
	2.	3.	4.	9.	12.	2.	3.	4.	9.	12.
				L4						
1–24	0	0	0	0	0	21	24	24	24	24
25–48	7	7	7	7	7					
49–72	21	16	16	16	16					
73–96	22	21	21	21	21					
				L7						
1–24	9	12	12	12	12	22	24	24	24	24
25–48	19	20	20	20	20					
49–72	22	22	22	22	22					
73–96	23	23	23	23	23					

THE ACTION OF ORGANIC GOLD COMPOUNDS ON PLEUROPNEUMONIA-LIKE ORGANISMS *IN VITRO*.

Only a small number of *in vitro* experiments on the action of gold salts on pleuropneumonia-like organisms have been undertaken, and when more favourable circumstances arise, further investigations are proposed. The results so far obtained show, however, that solganal B has both a bacteridical and bacteriostatic action on L4 *in vitro*. The protocols of one such experiment are given.

18.viii.39: Tubes containing 2 c.c. of blood broth were inoculated with 4 drops of a culture of L7. To the first set of 6 tubes before incubation was added solganal B to form dilutions from 10^{-1} to 10^{-6}; the second set of 6 tubes was incubated for 48 hours at 37° C., and thereafter solganal B was added to make the same dilutions from 10^{-1} to 10^{-6}. The results are shown in Table IX, where the figures indicate the number of mice out of 6 developing arthritis when injected into the pad of the feet with the cultures.

TABLE IX.—*The Effect of Dilutions of Solganal B on the Growth of L7 in Blood Broth when added before and 2 days after Incubation at 37°C.*

Dilution of solganal B.	Solganal B added before incubation. Living organisms present in days.						Solganal B added 2 days after incubation. Living organisms present in days.					
	1.	3.	5.	7.	9.	12.	2.	4.	6.	8.	9.	12.
10^{-1}	—	—	—	—	2	4	—	—	—	—	—	—
10^{-2}	3	3	3	5	5	5	—	—	2	4	4	4
10^{-3}	3	3	6	5	6	6	—	—	—	1	1	1
10^{-4}	—	—	—	—	3	3	—	—	—	—	—	—
10^{-5}	—	—	—	1	3	4	—	—	—	—	—	—
10^{-6}	2	2	2	4	5	5	—	—	2	5	5	5
Control	2	3	3	5	6	6	1	1	3	6	6	6

It will be seen that while solganal B is bacteriostatic and bactericidal, these actions vary with the dilution of the compound, the most effective dilutions being 10^{-4} and 10^{-5}. No explanation is forthcoming for this zone phenomenon, but it is of interest to note that a similar fact apparently holds good for the bactericidal action of organic gold salts on tubercle bacilli *in vitro*. Courmont, Gardère and Pichat (1932) and Pichat (1939) have found that with sanocrysin, allochrysin and solganal (aurothioglucose) dilutions of from 1/10,000 to 1/1,000,000 are bactericidal, but dilutions of from 1/500 up to 1/10,000 or above 1/1,000,000 are without action on tubercle bacilli.

DISCUSSION.

The results here recorded show that organic gold salts have a specific action in preventing the growth *in vivo*, and under certain conditions *in vitro*, of pleuropneumonia-like organisms isolated from rodents. The action of the gold salts is seen in preventing the development of arthritis in rats and

mice and of encephalitic symptoms in mice. Efforts to obtain pleuropneumonia-like organisms from animals in which the development of symptoms has been prevented by the action of gold has failed and such treated animals are not immune to re-inoculation. Arsenic, both in the tervalent form, as in neoarsphenamine, and in the quinquevalent form, as in acetarsol or tryparsamide, has a slight action on the pleuropneumonia-like organisms of rodents. Neoarsphenamine and acetarsol have previously been used, with apparently beneficial results, in the treatment of agalactia of sheep (Bridré, Donatien and Hilbert, 1928; Curasson, 1929) and pleuropneumonia of cattle (Witt, 1925).

Lithium antimonyl tartrate, sodium salicylate, sulphanilamide and sulphapyridine have no action on the pleuropneumonia-like organisms investigated.

The action of organic gold salts in preventing arthritis due to pleuropneumonia-like organisms in rodents naturally suggests a similarity to the action of the same salts in human rheumatoid arthritis. Unfortunately the causal agent of rheumatoid arthritis is still unknown. Our own efforts to grow a pleuropneumonia-like organism from the joint fluids of 4 cases of rheumatoid arthritis and from pericardial and pleural exudates of 2 other cases have entirely failed. For the present, therefore, no analogy can be drawn between the action of gold on the pleuropneumonia-like organisms of rodents and in rheumatoid arthritis of man.

SUMMARY.

1. Sulphanilamide, sulphapyridine, sodium salicylate and lithium antimonyl tartrate were without action on certain pleuropneumonia-like organisms isolated from rodents.

2. Neoarsphenamine was found to have a slight action in preventing the development of arthritis in mice due to the pleuropneumonia-like organism L7. Neoarsphenamine, acetarsol and to a less extent tryparsamide were slightly active in preventing the development of encephalitic symptoms in mice following intracerebral injection of L5 and L6.

3. Organic gold preparations were highly active in preventing arthritis in rats and mice and encephalitis in mice due to L4, L5, L6, L7 and the guinea-pig organism.

4. Organic gold preparations are bactericidal to pleuropneumonia-like organisms both *in vitro* and *in vivo*.

Our thanks are due to Miss Emmy Klieneberger for cultures of the L4 organism.

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A NEW BLOOD TEST AND ITS APPLICATION TO THE MICRO-DETERMINATION OF HÆMOGLOBIN.

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THE catalytic chemical tests for blood depend on the " pseudo-peroxidase " activity of the blood pigment, by means of which suitable substances, such as benzidine and gum guaiacum, are oxidized to coloured end-products by hydrogen peroxide. In this paper attention is drawn to the fact that the colourless reduced form of the oxidation-reduction indicator 2 : 6-dichlorophenol indophenol is a useful reagent for the detection of blood. In the presence of blood, this indicator is converted into the red oxidized form by hydrogen peroxide. The red colour produced in a positive test does not fade on standing, and the test is therefore preferable to the guaiacum test, in which rapid fading of the blue colour is a disadvantage in assessing the quantity of blood present. Also, on account of the stability of the oxidized form of 2 : 6-dichlorophenol indophenol it is possible to take the intensity of the red colour as a measure of the quantity of blood originally present. In this way the reaction has been extended to determine the hæmoglobin content of blood. The method is specially adapted to the estimation of hæmoglobin in minute quantities of blood, since the equivalent of 0·00025 ml. is sufficient. In this connection one application of the method is to the determination of hæmoglobin in the unused portion of the diluted blood taken for a blood-count. Assuming the blood has been diluted 200 times with saline for this purpose a measured sample of this suspension of cells is diluted another 10 times with water to make a 1 in 2000 dilution.

PROCEDURE.

Reagents.

(a) 0·2 *per cent. alcoholic* H_2O_2.—Add 0·5 ml. 30 per cent. H_2O_2 (Perhydrol) to 75 ml. alcohol.

(b) *Reduced indicator solution.*—Mix 0·2 g. 2 : 6-dichlorophenol indophenol with 120 ml. glacial acetic acid. Add 25 ml. 1 per cent. stannous chloride solution ($SnCl_2.2H_2O$) in 20 per cent. acetic acid. Stir until the dye dissolves, warming slightly if the dye is granular. Finally add 20 g. crystalline sodium acetate dissolved in 50 ml. water. Store the reagent in a dark stoppered bottle.

(c) *Stock hæmoglobin solution.*—Determine the hæmoglobin content of a sample of normal blood by the O_2 capacity method of Van Slyke and Neill (1924). Dilute the blood with 25 per cent. alcohol to give a solution of hæmoglobin containing 1·3 g. per 100 ml. Store the solution in a stoppered bottle in the refrigerator.

The Test for Blood.

Place 5 ml. of the specimen in a clean test-tube, add 2 ml. of glacial acetic acid and boil the mixture thoroughly. Cool the tube to room temperature and add 5 ml. of ether. Extract the blood pigment by inverting the tube about 12 times. Place 5–10 drops reduced indicator solution in a second test-tube, and add 2–3 ml. alcoholic hydrogen peroxide. Transfer 1–2 ml. ethereal extract from the first test-tube to the mixture of reduced dye and hydrogen peroxide in the second test-tube. A rose-red colour develops in the presence of blood. If no colour appears in 2 minutes, the test is negative.

The Micro-determination of Hæmoglobin.

Dilute the well-mixed specimen of blood 2000 times with water, using a carefully calibrated pipette. Prepare a working standard solution of hæmoglobin by diluting the stock 1·3 per cent. hæmoglobin solution 200 times with water, *e. g.* 0·5 ml. to 100 ml.

Pipette 2 ml. of the reduced indicator solution into two test-tubes labelled " standard " and " unknown ". Add 2 ml. of the alcoholic H_2O_2 and mix thoroughly, making sure that no H_2O_2 is left on the sides. Add 0·5 ml. of the standard and immediately afterwards 0·5 ml. of the unknown solution of hæmoglobin to the appropriate tubes and mix thoroughly. After 1 hour, add 10 ml. water to each tube, inverting several times to mix the contents. Compare the unknown with the standard in the colorimeter, using a green glass light filter to facilitate the matching.

$$\text{Calculation : } \frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 13 = \left\{ \begin{array}{r} \text{g. hæmoglobin per} \\ \text{100 ml. blood.} \end{array} \right.$$

When a blood low in hæmoglobin is encountered a more suitable dilution, *e. g.* 1 in 1000 or 1 in 500, should be made.

DISCUSSION.

As is usual in chemical tests for blood all apparatus must be scrupulously clean. In the test for blood, the mixture of H_2O_2 and reduced indicator should remain colourless for at least 10 minutes. In the determination of hæmoglobin a slight colour develops in the blank after standing for one hour, but the intensity of this colour is not sufficient materially to interfere with the straight line relationship existing between the intensity of colour and the concentration of hæmoglobin. This relationship is shown in Table I.

TABLE I.—*The Relationship between Colour and Concentration.*

Theoretical concentration.	Experimental result.
0·50	0·52
0·667	0·67
1·00 (Standard)	1·00
1·33	1·32
2·00	1·97

The factors which influence the rate of development of colour, and the stability of the blank, are the concentration of the alcoholic H_2O_2 and the pH of the medium. An increase in the concentration of H_2O_2 is followed by a more rapid rate of development of colour, but the final colour is no longer proportional to the concentration of blood pigment. Using less H_2O_2 the colour develops slowly. Changes in the pH of the reaction mixture have a pronounced effect on the stability of the mixture of hydrogen peroxide and reduced indicator, and therefore the pH is stabilized by the acetate/acetic acid buffer mixture in which the reduced indicator is dissolved.

A series of hæmoglobin determinations was carried out on specimens from human subjects by the above method and compared with the results obtained using Wu's (1922) modification of the acid hæmatin method. The same blood served as a standard in both methods. Some typical results are given in Table II.

TABLE II.—*A Comparison of the Present Method and Wu's Alkaline Hæmatin Method.*

Specimen.	g. hæmoglobin per 100 ml. blood.	
	Alkaline hæmatin method.	Reduced indicator method.
1	19·4	19·6
2	15·6	16·0
3	14·4	14·4
4	11·6	11·9
5	8·0	8·3
6	2·8	2·7

In the test for blood, extraction of the blood pigment by means of ether is adopted, since in common with similar tests, interference from iodides and other substances is avoided. The test is also rendered much more sensitive and reliable since powerful reducing substances, such as ascorbic acid, which prevent the development of colour in chemical tests for blood, are not extracted (Barrett, 1936). Nitrites are decomposed by boiling the acidified specimen. The limit of the test is reached with a 1 in 50,000 dilution of blood. A 1 in 10,000 dilution gives a definite response.

SUMMARY.

The reduced form of the oxidation reduction indicator 2 : 6-dichlorophenol indophenol is a useful reagent for the detection of blood. In the presence of H_2O_2 and blood pigment a red colour is produced. A response is obtained with a 1 in 50,000 dilution of blood.

The reaction has been utilized to determine the hæmoglobin content of minute quantities of blood. The equivalent of 0·00025 ml. is required for a determination.

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THE NUTRITION OF *STREPTOCOCCUS HAEMOLYTICUS.* GROWTH IN A CHEMICALLY DEFINED MIXTURE; NEED FOR VITAMIN B_6.

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HAEMOLYTIC streptococci are normally grown in broth containing meat extract, serum or blood. The study in this laboratory of the essential nutrients of the organism, which aims at interpreting the effects of such media in terms of definite substances they contain, began at a time when, though nothing was known of the nutrition of *Streptococcus haemolyticus*, a number of definite compounds had been found essential for the growth of other micro-organisms. These compounds were added on presumptive grounds to all media used, and

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the determination of further nutritive requirements was carried out in several stages by finding at each stage a medium which, though itself not adequate for growth, became so by the addition of relatively small quantities of natural substances. This addendum was then fractionated until its effect could be attributed to definite compounds in it. When identified these were added to successive media as pure compounds and another basal medium found, inadequate with this augmented group of known compounds, but adequate with a further addendum.

This process of growth on media containing progressively less unknown material has continued until we can now report growth of *Streptococcus haemolyticus* on a medium all components of which are known. The following summary gives the findings at each stage ; the later stages are given in detail in this paper.

1. Growth in Difco Bactopeptone in the presence of Mixture A (see below for details of media) required meat extract, the effect of which was replaced entirely by glutamine (McIlwain, Fildes, Gladstone and Knight, 1939).

2. Growth in HCl-hydrolysed casein in the presence of Group A and glutamine required a yeast or liver extract which was effectively replaced by pantothenic acid (McIlwain, 1939).

3. Growth in a mixture of amino acids and H_2SO_4-hydrolysed egg-albumin with Group A, glutamine and pantothenic acid (Medium I) required yeast extracts which were replaced by vitamin B_6 and a number of compounds, Group B.

4. Growth in a mixture of amino acids with glutamine, pantothenic acid, vitamin B_6 and Groups A and B (Medium II) required additional material from yeast which has not yet been identified, but which has been replaced by further groups of compounds C and D together with mercaptoacetate or certain other materials.

TECHNIQUE AND MATERIALS USED.

The test organism was a "Richards" strain of *Streptococcus haemolyticus* maintained at high virulence (McIlwain *et al.*, 1939) and taken for inoculation from an 18–24-hour growth on fresh broth agar. Growth in the experimental medium was at first diffuse, becoming granular and forming a precipitate within 24 hours of the commencement of growth.

Constituents of Media.

Amino acid basal medium was that described by Gladstone (1939) containing aspartic acid, valine, leucine, alanine, glutamic acid, *iso*leucine, phenylalanine, lysine, glycine, proline, hydroxyproline, tyrosine, arginine, histidine and buffer.

Group A consisted of the *Mixture A* (McIlwain *et al.*, 1939) of aneurin, nicotinamide, β-alanine, pimelic acid, uracil, cytosine, thymine, guanine, riboflavine, glucose and buffer with the further addition of $(NH_4)_2SO_4$ (1 g.), NH_4Cl (1 g.), methionine (0·05 g.), cystine (0·24 g.), $MgSO_4$ (0·2 g.), Fe $(NH_4)_2(SO_4)_2.6H_2O$ (0·05 g.) per 100 ml. It was prepared in the previous manner and filtered to sterilize.

Group B was an 0·02 *M* solution of serine (synthetic), threonine (synthetic), betain (synthetic), glucosamine (natural, boiled with conc. HCl and recrystallized) and inositol (natural, recrystallized), neutralized and filtered to sterilize.

Group C was an 0·02 *M* solution of ornithine (from natural arginine, recrystallized), citrulline (from arginine), β-hydroxyglutamic acid (prepared according to Harington and Randall, 1931) and asparagine (natural, recrystallized) neutralized and filtered to sterilize.

Group D was an 0·002 *M* solution of xanthine (natural), hypoxanthine (natural) and thiochrome (synthetic, from Dr. A. R. Todd) neutralized and autoclaved to sterilize.

Vitamin B_6 (adermin). The synthetic hydrochloride was given by Merck and Co., Rahway, N.J., initially through Dr. T. F. Macrae. An 0·005 *M* solution was autoclaved to sterilize.

Pantothenic acid was obtained according to Williams *et al.* (1938) from liver concentrates given by Dr. S. Smith of Burroughs Wellcome, Ltd., Dartford, Kent. A neutral solution estimated to contain 5×10^{-4} *M* pantothenate was sterilized by autoclaving.

H_2SO_4-hydrolysed egg albumin was prepared by autoclaving egg albumin (10 g.) with 10 per cent. v/v H_2SO_4 (100 ml.) at 130° for three hours. H_2SO_4 was removed by baryta, the solution neutralized and adjusted to 100 ml. For use in Medium I this was stirred with norite (10 g.) for two hours, filtered, and autoclaved to sterilize.

Yeast preparation A.—Baker's yeast was boiled with three times its weight of water for five minutes and filtered through asbestos-paper pulp. $Pb(CH_3COO)_2$ and $Hg(CH_3COO)_2$ were successively added in excess, maintaining p*H* 7 by the addition of NaOH. The precipitate was filtered off, excess metals removed from the solution by H_2S, this removed by air, the solution adjusted to p*H* 7 and a concentration equivalent to 0·25 g. yeast/ml., and autoclaved to sterilize.

Yeast preparation B was a cytolysate prepared according to Pirie's method (1930) for extraction of glutathione.

HCl-hydrolysed casein medium was that described by McIlwain (1939). The addition of arginine, as indicated in Table I, was found to accelerate growth under these conditions.

Media.

Medium I contained per tube of 10 ml. :

	ml.	
Amino acid basal medium . .	6	(autoclaved in tubes).
0·056 *M* phosphate buffer, pH 7·6.	0·8	mixed in bulk, immediately before testing, from sterile stock solutions and aliquots containing these quantities added to each tube with sterile precautions).
Group A	0·5	
*N*NaOH	0·1	
0·02 *M* glutamine . . .	0·25	
5×10^{-4} *M* pantothenate . .	0·05	
H_2SO_4 hydrolysed egg albumin .	0·2	
Inoculum containing *c.* 1000 org. .	0·1	
Solutions to be tested.		
Water to 10 ml.		

Medium II.—The albumin was omitted from Medium I, and in its place was added 5×10^{-3} *M* vitamin B_6 (0·05 ml.) and 0·02 *M* group B (0·2 ml.). For the experiments of Table IIIA, each amino acid of the basal medium was prepared as a sterile solution at 100 times its normal concentration, and 0·1 ml. added to the requisite quantity of buffer.

Medium III.—Medium II was modified by the addition of 0·02 *M* Group C (0·2 ml.) and 0·002 *M* Group D (0·02 ml.) and the omission of 0·4 ml. buffer.

TRANSFERENCE TO MEDIUM I (STAGE 3).

The hydrolysed casein used as basal medium while testing for pantothenic acid obviously consisted mainly of amino acids, and hence, even before pantothenic acid was identified, attempts were made to replace casein by the mixture of amino acids described by Fildes *et al.* (1936) as necessary for staphylococcus, with the additions found necessary by Gladstone (1939) for *B. anthracis*. The active material in yeast later identified as pantothenic acid was at that time added as a lead and mercury filtrate from a hot extract of yeast (yeast preparation A). Growth did not, however, take place under these conditions (Table I) but it was found possible to supply the major amino acid requirements in this manner, after which the medium required the addition of only a relatively small quantity of protein hydrolysate for growth to occur (C, Table I).

TABLE I.—*Necessity for Second Yeast Factor.*

Media containing Group A, glutamine, and the materials indicated. Concentrations are those in the final media ; ++ signs are roughly proportional to mass of growth.

			Growth after—	
			1–820 hours.	3 days.
A.	HCl hydrolysed casein with 10^{-4} *M* arginine	+ 0	0	0
	Do.	+ pantothenate 10^{-6} *M*	+++	++++
	,,	,, 0·2 $\times 10^{-6}$	++	+++
	,,	,, 0·04 $\times 10^{-6}$	+++	+++
	,,	,, 0·008 $\times 10^{-6}$	++	++
B.	Amino acid basal	+ 0	0	0
	,, ,,	+ H_2SO_4-hydrolysed egg albumin 0·2%, 0·04%	0	0
C.	,, ,,	Do. 0·2% + yeast preparation A 5%	+++	++++
	,, ,,	,, Do. 1%	+	+++
D.	,, ,,	,, 0·2% + pantothenate 10^{-5}–10^{-8} *M*	0	++
	,, ,,	,, 0·04% Do.	0	0

When pantothenic acid was identified and substantially pure concentrates could be used, little or no growth occurred even with the addition of albumin ; the yeast preparation evidently contributed to the medium other essentials

as well as pantothenic acid, and of these the hydrolysed egg albumin contained little.

The amino acid basal medium with H_2SO_4-hydrolysed egg albumin and pantothenic acid (Medium I) was accordingly used as basal for the attempted identification of this second factor of yeast. Examination of different sources showed that there was much more of the second factor in brewers' than in bakers' yeast or turnip. Na_2SO_4 extracts (McIlwain, 1939) had only 1/25th of the activity of extracts prepared by a few minutes' boiling. A hot extract of brewers' yeast was accordingly used as source for investigation of its further properties. The material was found to be adsorbed on norite, and advantage was taken of this to remove from the hydrolysed albumin in the basal medium traces of the second yeast factor which were causing slight delayed growth in control tubes (compare Table I, D, and Table II, A). The new factor was stable to autoclaving and to boiling in *N* acids and alkalis. When its further reactions had been investigated, comparison was made with compounds of known biological activity in yeast.

Parallelism was found between the present factor and vitamin B_6, which, in addition to being essential to rats, had been found necessary for growth of lactic acid bacteria (Möller, 1938), and to accelerate the growth of yeast (Eakin and Williams, 1939). When tried in place of the second yeast factor, vitamin B_6 caused growth (Table II), but this was delayed 24 hours or more in comparison with that obtainable with a yeast extract. Shortly after this discovery, vitamin B_6 was described as a growth factor for streptococcus by Hutchings and Woolley (1939).

TABLE II.—*Vitamin B_6 as Main Component of the Second Yeast Factor.*

		Growth after—		
		18–24 hours.	2 days.	3 days.
A.	Medium I	0	0	0
	,, + hot extract of brewers' yeast 0·1	++++	++++	++++
	,, Do. 0·02	++	++++	++++
	,, ,, 0·004%	+	+++	+++
	,, + vitamin B_6 10^{-5} *M* and over	0	+	+++
	,, ,, $0·2 \times 10^{-5}$ *M*	0	+	++
B.	,, ,, $2·5 \times 10^{-5}$ *M* + serine, 10^{-3} *M*	0	++	+++
	,, ,, ,, $0·2 \times 10^{-3}$ *M*	0	+++	+++
	,, ,, + threonine, 10^{-3} *M*	+	+++	+++
	,, ,, ,, $0·2 \times 10^{-3}$ *M*	0	++	+++
	,, ,, + betain, 10^{-3} *M*	0	++	+++
	,, ,, ,, $0·2 \times 10^{-3}$ *M*	0	+	+++
	,, ,, + glucosamine 10^{-3} *M*	0	+++	+++
	,, ,, ,, $0·2 \times 10^{-3}$ *M*	0	+++	+++
	,, ,, + inositol 10^{-3} *M*	0	+++	+++
	,, ,, ,, $0·2 \times 10^{-3}$ *M*	0	+	+++
C.	,, ,, + Group A 10^{-3} *M*	++	++++	++++
	,, ,, ,, $0·2 \times 10^{-3}$ *M*	+	++++	++++
	,, ,, ,, $0·04 \times 10^{-3}$ *M*	0	++	+++
	,, 0 ,, 10^{-3} *M*	0	0	0
Medium I without albumin	+ vitamin B_6, $2·5 \times 10^{-5}$ *M* + Group A 10^{-3} *M*	0	0	0

In the meanwhile, a number of other compounds which had been found to influence streptococcus growth in this and other laboratories were also tested in place of the second yeast factor, irrespective of any similarity in properties. In complete absence of vitamin B_6, none of these compounds caused growth. With, however, H_2SO_4-hydrolysed egg albumin which had not been treated with norite and consequently contained a little B_6, increased growth was observed in some cases. A number of these compounds were consequently tested in the presence of an optimal quantity of B_6 with the intention of accelerating growth. The results in Table II (B) show the effects of five compounds tested at this stage which improved growth. Serine, threonine, and inositol were chosen on general grounds as being involved in the nutrition of other organisms; betain and glucosamine are among a list of compounds described by Rane and Subbarow (1938) as improving the growth of *Streptococcus haemolyticus*. Urea, guanidine, creatine, arginine and other compounds were without action. It will be noted that the effect of the active compounds (Group B) under these conditions is only to increase growth; they cannot (Table II, C) cause it in absence of B_6 or of albumin. The compounds of Group B, singly or collectively, do not at this stage afford the all or none effect usually associated with a definite nutritional factor; this may be due to presence of suboptimal quantities of these or similar compounds in the albumin preparation. Table II (C) nevertheless shows that the albumin contributes a distinct nutritional factor.

REPLACEMENT OF ALBUMIN BY KNOWN COMPOUNDS (STAGE 4).

A. *Some Amino Acid Requirements.*

The effect of the yeast addendum of Stage 3 was thus replaced by vitamin B_6, serine, threonine, glucosamine, betain and inositol; these, added to the amino acid basal medium with Group A, constituted a deficient medium (Medium II) requiring the addition of H_2SO_4 hydrolysed egg albumin for growth in less than six to seven days. Some properties of the albumin factor were examined. It was found stable to autoclaving and to mild treatment with acids and alkalis; it was destroyed by nitrous acid, extracted slowly from aqueous solution by butyl alcohol and precipitated by Hg^{++} under Neuberg's carbonate-alcohol conditions. These properties, and a chance observation that a batch of the amino acid basal medium, autoclaved at a pH of >10, permitted growth in two to three days, led to a consideration of the specific amino acid requirements of streptococcus.

Table III shows first the effect of omitting each amino acid in turn. In most cases this prevented growth, but with glutamic acid and arginine the effect was only to reduce growth. With glutamic acid this is understandable, as glutamine is added to the medium in quantities about equal to its normal glutamic acid content. Effect of the lack of arginine is more pronounced, but the growth without it may be explained in terms of the observation of Hills (1939) that it was the only amino acid which the present strain of streptococcus was capable of metabolizing at an appreciable speed. Nevertheless, growth was best in the presence of these amino acids, which were

TABLE III.—*Some Amino Acid Requirements.*

	Growth (days).								
	1.	2.	3.	4.	5.	6.	7.	8.	10 and 12.
Medium II (duplicate)	0	0	0	0	0	+	++	+++	+++
A. *Lacking :*									
Glutamic acid	0	0	0	0	0	0	+	+++	+++
Arginine	0	0	0	0	0	0	+	++	++
Aspartic acid, valine, leucine, alanine, *iso*leucine, phenylalanine, lysine, proline, tyrosine, *or* histidine	0	0	0	0	0	0	0	0	0
B. *With addition of* 0·001 *M :*									
Ornithine	0	0	0	0	++	+++	+++	+++	+++
Citrulline	0	0	?	++	+++	+++	+++	+++	+++
β-hydroxyglutamic acid	0	0	0	0	++	+++	+++	+++	+++
α-aminobutyric acid	0	0	0	0	0	+++	+++	+++	+++
Norleucine	0	0	0	0	0	0	0	?	+++
β-hydroxyglutamic acid + citrulline + ornithine	0	0	?	+++	+++	+++	+++	+++	+++
Albumin, 0·2%	++	++++	++++	++++	++++	++++	++++	++++	++++

accordingly left in the medium. In view of the results of Gladstone on the amino acid requirements of *B. anthracis* (1939), lack of growth of streptococcus following the omission of a single amino acid cannot be taken to mean that this is essential; a different group of amino acids, also without the same amino acid, may permit growth. These results, however, imply that such amino acids, if not essential, are involved in the metabolism of streptococcus.

As serine, threonine, methionine, cystine and tryptophane are present in groups added to Medium II it contains 19 α-amino acids. Nevertheless the further separate addition of α-aminobutyric acid, β-hydroxyglutamic acid, ornithine and citrulline accelerated growth (Table III, B). These effects, especially marked in the case of the last two compounds, were additive to a small degree only, and growth with all four together remained two to three days behind that obtainable with albumin. These compounds, together with asparagine, which had been found to accelerate growth when added to a medium lacking B_6, but containing un-norited albumin (p. 27), were added as Group C in further experiments. The β-hydroxyglutamic acid was a synthetic specimen containing all four possible isomerides.

B. *Glutathione and Materials with Related Effects.*

It was evident that at this stage growth could be accelerated and increased in mass by the addition of a number of compounds in a manner which, unlike the normal effect of a growth essential, was not specific to any one. Similar effects have been described by Rane and Subbarow (1938), Subbarow and Rane (1939), and Woolley and Hutchings (1939). The majority of compounds found active by those workers have already been incorporated in the present medium, which differs from the media used by the above workers in being composed entirely of known compounds; other substances described as active were accordingly tried. The effect of addition to Medium II of xanthine, hypoxanthine, thiochrome (Group D), glutathione and vitamin C is shown in Table IV. The most marked effect was obtained with glutathione; this was accelerated by the further addition of the other compounds, when growth was superior to that obtained with the albumin preparation.

TABLE IV.—*Further Additions to Medium II.*

	Growth (hours and days).				
	18.	25.	2.	4.	6.
Medium II	0	0	0	0	?
,, + Groups C and D, 0·001 *M* .	0	0	0	0	0
,, + Group D, 0·0001 *M* . .	0	0	0	0	0
,, + natural glutathione, 0·001 *M*	+	+	+++	+++	+++
,, + vitamin C, 0·001 *M* . .	0	0	++	+++	+++
,, + Groups C and D + natural glutathione, 0·001 *M* .	+++	+++	++++	++++	++++
,, + H_2SO_4-hydrolysed albumin, 0·2%	++	++	++++	++++	++++
,, + marmite, 0·2% . .	+++	++++	++++	++++	++++

As Groups C and D had caused no growth under the conditions of Table IV (Group D retards growth), they were included in the basal medium, forming medium III, and the effect of glutathione further investigated (Table V).

TABLE V.—*Further Additions to Medium III.*

		Growth (hours and days).				
		18.	24.	2.	3.	4.
A.	Medium III	0	0	0	0	0
	,, + cysteine, 2×10^{-3} *M*	0	0	?	+++	++++
	,, ,, $0{\cdot}4 \times 10^{-3}$ *M*	0	0	0	?	+++
	,, + cysteine, 2×10^{-3} *M* + glutamic acid, 2×10^{-3} *M* + glycine, 2×10^{-3} *M*	0	0	0	+++	++++
	,, + mercapto-acetate, 5×10^{-3} *M*	++	+++	+++	+++	+++
	,, ,, 10^{-3} *M*	0	0	0	+++	+++
	,, + mercaptosuccinate, 5×10^{-3} *M*	+	++	++	++	++
	,, ,, 10^{-3} *M*	?	?	+	++	++
	,, + homocysteine, 5×10^{-3} *M*	++	+++	+++	+++	+++
	,, ,, 10^{-3} *M*	0	0	+	++	++
B.	,, + natural glutathione, $1{\cdot}3 \times 10^{-3}$ *M*	++	+++	++++	++++	++++
	,, ,, ,, $0{\cdot}6 \times 10^{-3}$ *M*	?	+++	++++	++++	++++
	,, ,, ,, $0{\cdot}3 \times 10^{-3}$ *M*	0	0	++++	++++	++++
	,, ,, ,, $0{\cdot}1 \times 10^{-3}$ *M*	0	0	?	++++	++++
	,, + synthetic glutathione $1{\cdot}3 \times 10^{-3}$ *M*	0	0	++++	++++	++++
	,, ,, ,, $0{\cdot}6 \times 10^{-3}$ *M*	0	0	+++	++++	++++
	,, ,, ,, $0{\cdot}3 \times 10^{-3}$ *M*	0	0	?	++++	++++
	,, ,, ,, $0{\cdot}1 \times 10^{-3}$ *M*	0	0	0	+	++++
	,, + yeast B 2	+++	+++	+++	++++	++++
	,, ,, 0·4	++	+++	+++	++++	++++
	,, ,, 0·08	++	+++	+++	+++	++++
	,, ,, 0·016%	+	+	++	++	+++
	,, + ,, Cu′ ppt. equivalent to 50	+++	+++	+++	++++	++++
	,, ,, ,, ,, 10	0	0	++	+++	+++
	,, ,, ,, ,, 2	0	0	0	+++	+++
	,, ,, ,, ,, 0·4%	0	0	0	++	+++
	,, + ,, Cu′ solution equivalent to 2	+++	+++	+++	++++	++++
	,, ,, ,, ,, 0·4	++	+++	+++	++++	++++
	,, ,, ,, ,, 0·08	0	?	+++	+++	+++
	,, ,, ,, ,, 0·016%	0	0	+++	+++	+++

The component amino acids of glutathione—glycine, glutamic acid and cystine—are present in the basal medium, but increased amounts of cystine or cysteine were sufficient to cause growth in two to four days. Increased quantities of glutamic acid or glycine had no effect, or a deleterious one. Other –SH compounds were therefore investigated, and mercapto-acetate, mercaptosuccinate and homocysteine all found to cause growth (Table V). The effect was evidently a non-specific one, and was indeed given by vitamin C and by reduced iron and sodium sulphide (*cf.* Woolley, 1939).

c. *Further Nutritional Requirements.*

Although the aim of growing *Streptococcus haemolyticus* in a medium of known composition has, in a sense, been realized by the above growth in the presence of e.g. mercapto-acetate, there are important reservations. Firstly, not all the compounds used are of synthetic origin, and, though all have undergone careful purification, the possibility remains of their being contaminated with small quantities of highly active materials. It is also necessary, especially in the cases of unspecific effects of the type encountered here, to consider which (if any) of the effective compounds is involved in nutrition of the organism under its normal conditions of growth.

New problems were immediately encountered in investigating these points. As the amounts of –SH compounds necessary for growth are unusually high, they were first investigated. The most active of them, glutathione, was replaced by a synthetic specimen obtained from Dr. V. du Vigneaud. This was found less active than material prepared from yeast (Table V, B); growth of equal mass was obtained only after one to two days' further growth.

When yeast itself (yeast preparation B), the source of the more active glutathione, was examined, it was found approximately 100 times more active than would be expected from its glutathione content. Separation of the glutathione was performed according to Pirie (1930); material recovered from the cuprous precipitate showed the same activity as purified natural glutathione, i.e. 1/100th of the original extract. The active filtrate contained very little S–S or SH. Thus the activity of yeast at this stage is not primarily due to its glutathione content, but to a further factor. Using Medium III as basal, other natural sources were examined for the active material. The albumin preparation was not very active, probably on account of destruction of the material during hydrolysis, for a more mildly HCl hydrolysed preparation was much more active. Similarly hydrolysed zein was equally active, edestin and casein less so. The most active sources were yeast and marmite. Activity was not proportional to total SH determined roughly by nitroprusside after reduction. The material has not been identified, but the results are recorded at this stage on account of the success obtained with mercapto-acetate *et al.* as substitutes.

DISCUSSION.

These results have illustrated types of nutritional requirement different from that to which the term "growth factor" was first applied. Growth factors such as uracil, nicotinic acid, or vitamin B_1 for staphylococcus; glutamine and

probably vitamin B_6 and pantothenic acid for streptococcus can be replaced, if at all, only by compounds of very closely related chemical structure, and their effect remains equally important in the presence of excess of other growth requirements of the organism. On the other hand, as was first shown by Woolley (1939), the effect of glutathione can be reproduced by mercapto-acetic acid, vitamin C, and by reduced iron with sodium sulphide (to which the present results add mercaptosuccinate, homocysteine and probably more active compounds of yeast). Woolley indicated that these could be considered to have in common a reducing action.

Glucosamine, betain, inositol, asparagine and certain amino acids represent a third type whose individual effect was only definitely seen in presence of suboptimal amounts of other factors—of B_6, or of the SH-replaceable factor. They are structurally entirely unrelated. They could possibly be functionally related in the manner in which vitamin B_6 and fatty acids appear to be in the nutrition of the rat (Birch, 1938). These different types of growth requirement are not clearly defined, but are categories somewhat arbitrarily made in the nutritional requirements of an organism, which can vary from absolute dependence upon a material (when it cannot be synthesized by the organism) to complete indifference (when it can be made in excess in normal growth). Also, conditions of growth may change the effect of a given compound from one class to another; e.g. the requirement of streptococcus for glutamine is much more strict in the presence of a small inoculum than of a large one, when it is replaceable by glutamic acid; and if the medium contains traces of glutamine insufficient to allow growth, glutaric acid, α-oximinoglutaric acid or pyrrolidone-α-carboxylic acid, which are normally inactive, can appear as growth factors (McIlwain, unpublished).

These results make it especially important to consider which of a group of such compounds are of importance in growth of the organism under its natural conditions. This consideration, and the recognition of different degrees of necessity for nutritional factors, have not been emphasized by recent workers on streptococcus nutrition. As knowledge of nutritionally and metabolically important compounds grows, it becomes more profitable to attempt to replace an unknown nutritional factor by the many materials biologically important in other connections than to fractionate and identify the unknown factor by purely chemical methods. In the present work both methods have been used simultaneously; but when a previously known compound has been found active, qualitative and if possible quantitative comparisons have been made with a number of natural sources of growth-promoting material.

The unspecific effects of Groups B, C and D are difficult to investigate in this manner. Although all these compounds have been included in the basal medium used for testing for the SH-replaceable factor, opinion must be reserved as to their ultimate importance in streptococcus nutrition. Unlike the major components, their effect has not yet been demonstrated in the presence of excess of all other natural nutritional factors, as the SH-replaceable factor of yeast has not been obtained in pure condition. With mercapto-acetate or glutathione in place of this factor (when auxiliary needs may be different) Groups B, C and D can, however, collectively strictly control growth (Table VI).

TABLE VI.—*Growth-controlling Effect of Auxiliary Components.*

Inadequate basal medium = amino acid basal medium + Group A + glutamine + pantothenate + vitamin B_6 in the quantities used in Medium III.

			Growth (hours and days).			
			18.	24.	2.	7.
Inadequate basal medium	+ mercapto-acetate, $5 \cdot 10^{-3}$ *M*		0	0	0	0
,, ,,	+ natural glutathione, 2 10^{-3} *M*		0	0	0	0
,, ,,	+ yeast B, 2%		+++	+++	++++	++++
,, ,,	+ natural glutathione, $2 \cdot 10^{-3}$ *M* + Groups B, C and D;					
		0·02, 0·02, 0·002 *M* 1·0	++	+++	++++	++++
,, ,,	,, ,,	Do. 0·3	+++	++++	++++	++++
,, ,,	,, ,,	,, 0·1 ml.	+	++	++++	++++
,, ,,	,, ,,	+ yeast B, 10, 3 or 1%	+++	++++	++++	++++
,, ,,	+ Groups B, C and D; 0·1, 0·3 or 1·0 ml.		0	0	0	0

The fact that Rane and Subbarow (1938) and Subbarow and Rane (1939) have shown the effect of several of these compounds under different conditions supports, but does not prove their independent necessity.

A full investigation of the amino acid and auxiliary requirements of streptococcus has been left until optimal conditions of growth can be obtained with synthetic materials. The results of p. 32 are not considered final, and instances of mutually inhibitory effects of amino acids in growth have already been observed. It is not assumed from the present results that the SH-replaceable factor in yeast is necessarily an SH compound, nor that it entirely replaces all SH compounds; it may be a compound of the Group C or D type in the presence of which SH compounds are either unnecessary or required in much lower concentrations.

The organism has been subcultured 8 successive times in Medium III with the addition of (*a*) *M*/200 mercaptoacetate and (*b*) *M*/500 natural glutathione, without change in its type or mass of growth. Suspensions containing 10 or 1000 organisms from the previous day's growth were used as inocula.

SUMMARY.

Streptococcus haemolyticus has been grown in a medium containing some 40 known compounds. Different degrees of nutritional requirement have been encountered; many compounds are absolutely essential for growth, others replaceable by compounds of like structure or chemical properties, and others interchangeable for compounds entirely unrelated in chemical properties and structures. Growth in the chemically-defined medium is practically optimal, but at least one group of compounds can be replaced by more active naturally occurring material which has not yet been identified.

I have pleasure in acknowledging my indebtedness to Dr. P. Fildes, who, as head of the Department, is responsible for the organization which made this work possible; to Dr. B. C. J. G. Knight, who took part in the initial stages of the investigation; to all members of the Department who have made helpful suggestions, and to the several persons mentioned in the text who have given some of the materials used.

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THE MODE OF ACTION OF SULPHANILAMIDE.

WITH SPECIAL REFERENCE TO A BACTERIAL GROWTH-STIMULATING FACTOR ("P" FACTOR) OBTAINED FROM *BR. ABORTUS* AND OTHER BACTERIA.

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AN investigation started two years ago into the sensitivity of *Br. abortus* to sulphanilamide quickly revealed that sulphanilamide compounds had a bacteriostatic and bactericidal action in defibrinated blood, serum, nutrient broth and saline. It was shown also that after the administration of sulphanilamide by mouth, the blood from man, rabbit and guinea-pig was highly bactericidal.

At the same time, therapeutic experiments based partly on survival rates in the guinea-pig showed the efficacy of sulphanilamide in relatively high doses in overcoming *Br. abortus* infection. Using different criteria of cure a similar therapeutic effect in guinea-pigs has been reported by Wilson (1939) and Chinn (1939). An account of the therapeutic experiments, in which, incidentally, it was shown that general gamma irradiation of the guinea-pig completely inhibited the therapeutic activity of sulphanilamide, is to be published separately.

Meanwhile, the sensitivity of the Brucella group in vitro was reported by Francis (1938), Chinn (1939) and Meneffe and Poston (1939). These workers only observed the bacteriostatic effect for short periods (two to four days), and as will be seen, such short experimental times give an inaccurate idea of the ultimate fate of the inoculated organisms, and the bactericidal potency of sulphanilamide. Some degree of bacteriostasis always occurs and is either followed by sterilization, or at some later period of days or even weeks, by rapid growth. The factors determining whether *Br. abortus* will at some stage grow readily in sulphanilamide have been particularly investigated in the present work. No detailed account of the earlier work will be given, since the protocols are too extensive, but use will be made of typical findings, considered to illustrate the more fundamental facts arising out of this work.

The explanation of many confusing results obtained in this work, and appearing in the published details of others, concerning the very variable potency of sulphanilamide in different media, and with different sized bacterial inocula, became clear when it was shown that the final bacterial density

reached in the preliminary growth phase, was the factor determining whether the organism would survive in sulphanilamide.

The nature of the medium affected the issue only by conditioning the rate of growth. Gay, Clark, Street and Miles (1939) have also demonstrated the relation between the growth-promoting properties of the medium and the growth-rate of streptococci in sulphanilamide solutions.

This quantitative relationship between the bacterial density and the action of sulphanilamide is particularly well studied with *Br. abortus*, since it is a relatively slow-growing organism which may survive for a much longer time in the presence of sulphanilamide than most bacteria do. The changes in bacterial population can be analysed more easily than when an organism whose life-cycle is short, and which readily succumbs to the lethal action of sulphanilamide or dies out in fluid media in 48 hours or so, is used.

In the early stages of this work the conviction gradually developed that *Br. abortus*, under certain conditions, was capable of releasing some factor into the medium which inhibited the action of sulphanilamide, and many attempts were made to obtain clear evidence of its presence, and to extract it directly from bacteria.

Good circumstantial evidence of its presence was soon found, and later it was extracted in large amounts from broth cultures of *Br. abortus* and their filtrates. Progress was delayed by the unsuspected presence of growth-inhibiting agents in the early preparations, which often masked completely the effect of the anti-sulphanilamide factor.

Direct extraction of bacteria was then tried, and it was found that when *Br. abortus* was allowed to autolyse in water the filtrate contained some factor which inhibited the action of sulphanilamide on *Br. abortus* and other bacteria. The early preparations were relatively weak merely because the density of the suspensions used was too low and the time allowed for autolysis too short.

At this stage Stamp (1939) showed that a fraction could be isolated from a Group C haemolytic streptococcus, and partially purified or at least greatly concentrated, which antagonized the bacteriostatic action of sulphanilamide on the strain in question. He showed that the action was not specific to the organism, since it was exerted against the bacteriostatic effect of sulphanilamide on a Group A streptococcus, while a fraction from the Group A strain was active when tested with the Group C strain. The substance was shown to be extractable with N/25 NH_4OH, but not with water or N/25 HCl. It was shown to be resistant to boiling, and to the action of N/25 HCl. The concentrated preparations contained a mixture of substances of low molecular weight, including free amino-acids and were free from protein. In the discussion of these findings it was suggested that the substance was either an essential nutritive factor, or perhaps a co-enzyme, which was rendered unavailable or inactive by sulphanilamide.

When this method was applied to *Br. abortus* and other bacteria, preparations rich in an anti-sulphanilamide factor were obtained, but even after two extractions with dilute NH_4OH, simple autolysis in water yielded further large amounts of the factor. On returning later to the simple method of autolysis it was found that the method was superior to that of NH_4OH extraction, as it released larger amounts of the anti-sulphanilamide factor and the

extracts contained much less solid matter. As there is no qualitative difference in the action of the different extracts the earlier results obtained with ammonia extracts are mainly dealt with in this paper.

It was next shown that this anti-sulphanilamide factor had a powerful growth-promoting effect on *Br. abortus* and many other bacteria.

It is hoped to show in this very condensed account that the experimental facts presented have a direct bearing on the mode of action of sulphanilamide on bacterial growth, and that the growth-promoting factor is possibly a fundamental, non-specific agent, in bacterial reproduction.

All the experimental facts cited without qualification have been repeatedly confirmed, but space allows only a small fraction of the evidence being given.

Certain abbreviations are used in the text, namely : Su for sulphanilamide, B : Su ratio $\frac{\text{(Number of bacteria per ml.}}{\text{(Mgs. of sulphanilamide per ml.}} \times 10^5$), a purely arbitrary figure, useful in comparing the changes in bactericidal potency of Su at varying concentrations and in different media. " P " factor (pullulation or proliferation factor)—the bacterial growth-stimulating agent present in the extracts from *Br. abortus* and other bacteria used in this work.

METHODS.

The strain of *Br. abortus* was originally obtained from a patient with undulant fever, after culturing the blood in liver broth in an atmosphere of 10 per cent. CO_2. It grew well in air after a few subcultures, and has shown no alteration in colonial form, growth characteristics or virulence in animal tests, after repeated sub-cultures on glycerine agar for over two years.

A saline suspension from a two-day glycerine agar culture was matched against an opacity tube, and further dilutions made in nutrient broth. Originally viable counts (0·02 ml. volumes of serial bacterial dilutions were mixed with 10 ml. melted agar and the total number of colonies developing in the plates after five days were counted) were made with a suspension corresponding in density to a standard opacity tube. This tube was afterwards used for matching the initial suspensions, and standard 50 dropper pipettes were used throughout in making further bacterial dilutions and inplants from these. The term " inoculum " always refers to the number of organisms per ml. of the medium and not to the actual number implanted.

The required Su dilutions were made in nutrient broth (in which it is considerably more soluble than in saline) and autoclaved before used. Autoclaving does not affect the Su concentration, though there is a slight fall if the solution is exposed on the laboratory bench to daylight for some time (e.g. 15 per cent. loss in four weeks in a 1 : 1000 solution). Where not otherwise stated, p-amino benzene sulphonamide was the Su compound used. Sulphapyridine (M. & B. 693) and disodium para (7 phenylpropylamino) benzene sulphonamide -a, 7-di-sulphonate (Soluseptasine) were used less frequently since their effects were precisely similar to p-amino benzene sulphonamide, though differing in degree, sulphapyridine being somewhat more potent both in blood and broth, and soluseptasine very much less so.

All fluid media, except blood, were pipetted in 5 or 10 ml. amounts into

25 ml. screw-cap bottles. Care was taken to avoid evaporation, as it was often necessary to make repeated sub-cultures for as long as six to eight weeks. In earlier experiments 0·5 ml. amounts in 2 ml. corked tubes were used. Growth was less vigorous (apparently due to the smaller air space) and contamination (from the corks) more frequent.

Contamination in Su solutions was rare except when extracts containing "P" factor were present. The usual contaminants were *B. subtilis*, *Staph. albus* and diphtheroids, all of which, after preliminary bacteriostasis, grew well in Su-containing media.

Blood was defibrinated and used in 0·5 ml. amounts in 2 ml. corked tubes, which were rotated at 37° C. for 24 hours, and then left stationary. The appropriate Su dilutions were made by adding saline solutions of Su from a 50-dropper pipette.

The rate of growth in *Br. abortus* cultures was determined from the number of colonies developing from loop explants on glycerine agar and by the degree of turbidity developing in broth cultures. Turbidity observations and sub-cultures were made daily (and occasionally at intervals of a few hours on the first day) for periods up to 30 days and sometimes at less frequent intervals up to three months. Viable counts were made only in selected experiments, or if needed as a check on the plate cultures. Many thousands of sub-cultures were necessary in the course of the work, and rapid methods of determining the rate of bacterial growth were therefore essential. Turbidity estimations were visual and the standards arbitrary, + representing the first trace of turbidity, and ++++ the maximum turbidity possible.

A standard technique for plate-subcultures was used throughout. Serial strokes of the same length, using a standard loop, were made. This method, as checked by accurate viable counts, gave, at least in the case of *Br. abortus*, a reliable index of variations in the viable population. In fact, owing to the great heat-sensitivity of Su-treated organisms, the results of the viable counts suggested sterility at a stage in bacteriostasis when the direct stroke cultures were still positive. The Su contained in the explant diffuses out into the agar, and so long as reasonably thick plates are used, no error ever arises from a prolongation of bacteriostasis. Explants from experimental cultures, when greatly diluted in fluid media, always failed to grow if, at the same time, there was no sign of growth on plate cultures.

The plates must be observed up to the 9th day, for further colonies may develop up to, but not usually after, this time.

The following notation is used in the tables for the final number of colonies developing from stroke cultures. The roughly corresponding range of viable bacteria as determined experimentally is given.

Notation.	Organisms per ml.
50 (actual number of colonies stated)	$10^2 - 2 \times 10^4$
+	$2 \times 10^4 - 2 \times 10^5$
++	$2 \times 10^5 - 2 \times 10^6$
+++	$2 \times 10^6 - 2 \times 10^7$
S.C. (semi-confluent)	$5 \times 10^6 - 10^7$
C. (confluent)	2×10^7 and above

Testing for the presence of "P" factor.

The method naturally depended upon the object of the experiment. A reasonable estimate of the anti-Su potency can be obtained by using a fixed inoculum and various Su dilutions, or conversely, a range of different inocula and one dilution of Su, though for preliminary tests combinations of the two methods were often used. A very weak extract may not be effective with an inoculum of 2000 in 1 : 10,000 Su, whereas in the presence of a very potent extract an inoculum of 2 in 1 : 500 Su may grow out.

For routine tests the following method was adopted : An inoculum of 200 (*Br. abortus*) per ml. in 5 ml. of medium, consisting of 4·5 ml. 1 : 10,000 Su in broth plus 0·5 ml. of crude extract, was used. With concentrated extracts an amount equivalent to 0·5 ml. of the crude extract was used. All were set up in duplicate, with controls containing 0·5 ml. of saline. Out-growth, no matter how long delayed, indicates the presence of "P" factor, for even an inoculum of 2000 under these conditions very rarely grows out without added "P" factor.

If the extract is not sufficiently active to produce ultimate progressive growth, some activity may be revealed by a longer survival time as compared with control cultures. The relative potencies of active extracts may be roughly assessed by the number of days elapsing before turbidity (+) appears in the medium.

In testing for the pure growth-promoting effect of "P" factor as distinct from the anti-Su effect, small inocula (usually 2 or 20) in 4·5 ml. of broth or 0·9 ml. of blood plus 0·5 ml. or 0·1 ml. of extract were used. Using *Br. abortus* in fluid media the time of appearance of turbidity and the growth on plate sub-cultures gives reliable qualitative data, so strong is the effect. For exact tests, direct colonial counts of appropriate dilutions were made after 24 hours' incubation.

For preliminary tests on a series of different bacteria the extract was dissolved in nutrient agar (in an amount equivalent to 10 per cent. of crude extract), and explants made on to it from appropriately diluted cultures. This simple method often revealed unmistakeably increased proliferation judging by colonial size and the extent of spread, as compared with controls.

If the test organism (e.g. *haemolytic streptococcus*) only grows feebly in plain broth the activity of an extract may be shown by the development of turbidity. Control cultures remain clear.

Preparation of bacterial extracts.

The extracts used in these experiments were mainly prepared with various modifications, by the methods described by Stamp (1939). Towards the end of this work a method of obtaining still more potent extracts from *Br. abortus* was devised. This method, which involves preliminary autolysis, is being elaborated and will be described in detail in a later paper.

Ammonium chloride, even in the weak concentration (roughly M/25) present in the ammonia extract after neutralization, reduced the growth-rate of *Br. abortus*. Its presence was sufficient to conceal the presence of "P" factor in weak extracts. The NH_4OH extract was therefore boiled under low pressure

(which removed most of the ammonia), and was not brought to pH 4–5 as in the first stage of Stamp's method.

Similar methods, with minor modifications, were used for preparing extracts from other bacterial species and from organic material.

Crude extract (AE)a.

The organisms (dry weight 2–3 g.) from a seven-day growth of *Br. abortus* on glycerine agar (20 Roux bottles) are washed off with about 250 ml. of water, centrifuged down, and resuspended in 60 ml. of N/25 NH_4OH overnight. The suspension is shaken continually during the day and incubated at 37° C. overnight. After 48 hours of this treatment the bacteria are again centrifuged and the supernatant fluid concentrated to about 40 ml. by boiling at 35–39° C. Such extracts are practically neutral, but minute amounts of N/25 HCl may be required to adjust the pH to 7·4. It is then filtered through a bacterial filter (Seitz).

Crude extract (AE)b.

The organisms are then resuspended in 60 ml. of N/25 NH_4OH and the same process repeated.

Concentrated extract (AC)a.

About 2 ml. of the crude extracts are set aside for tests. The two extracts are pooled, concentrated to about 40 ml., and nine volumes of absolute alcohol added. After standing overnight in the ice-box the precipitate (which is quite inactive) is filtered off, and the filtrate evaporated nearly to dryness. The residue (dry weight roughly 250 mg.) is dissolved in about 15 ml. of distilled H_2O. Some inactive, insoluble material remains.

Crude extract (AE)c.

The bacterial sediment [after filtering off (AE)b] is now resuspended in 50 ml. of distilled H_2O and incubated for nine days at 37° C., shaking occasionally to resuspend it. The bacteria are centrifuged down and the supernatant fluid filtered.

Concentrated extract (AC)b.

The bulk of (AE)c is evaporated down to about one-third of the original volume ; nine volumes of alcohol are added, and after standing overnight in the ice-box the precipitate is filtered off, and the filtrate evaporated almost to dryness and dissolved in 10 ml. of H_2O. This extract contains from 1–3 mg. of solid material per ml.

The anti-Su and growth-promoting potency of these extracts increases progressively from (AE)a to (AC)b. (AE)a contains distinctly less " P " factor than (AE)b, and the 90 per cent. alcohol-soluble fraction (AC)a roughly five to ten times as much as (AE)b. The crude extract (AE)c, obtained after autolysis, is much more potent than (AE)b, whilst (AC)b contains at least twice as much " P " factor as (AC)a.

THE RELATIONSHIP BETWEEN THE INITIAL INOCULUM AND THE BACTERICIDAL ACTION OF SULPHANILAMIDE.

That a particular concentration of Su was not bacteriostatic or bactericidal to more than a certain number of bacteria, varying with the species, has been noticed by several observers (e.g. McIntosh and Whitby, 1939), but its significance does not appear to have been stressed. This relationship was observed in early experiments to be very close, so that, for instance, an inoculum of 2000 in 1 : 10,000 Su showed, after a preliminary growth phase, progressive bacteriostasis, and final sterilization, whereas an inoculum of 5000 after preliminary bacteriostasis grew out at a progressively increasing rate. This precise relationship was later defined even more exactly, so that, under the particular experimental conditions used, it was possible to forecast that an inoculum of 3000 would finally grow out vigorously. If the size of the inoculum is kept constant, the weaker the concentration of Su, the more rapid the growth, but even high dilutions of Su produce a measurable diminution of the initial growth rate as compared with controls. This quantitative relationship will be better appreciated from the experimental details given in the tables (see Table I).

TABLE I.—*Relation between Sulphanilamide Concentration and Survival of* Br. abortus *in Nutrient Broth. Viable Count and Growth on Plate from Loop Explant Compared* (*Inoculum of* 200,000 *per ml.*).

Days.	1/200 Su.		1/1000 Su.		1/5000 Su.		1/20,000 Su.		Broth alone.	
	Bacteria per ml. 1000.	Loop explant.	Bacteria per ml. 1000.	Loop explant.	Bacteria per ml. 1000.	Loop explant.	Bacteria per ml. 1000.	Loop explant.	Bacteria per ml. 1000.	Loop explant.
1	225	+	1,440	++	2,900	+++	6,300	C	10,530	C
2	0	+	6,480	+++	10,400	C	27,000	C	∝	C
3	0	+	2,250	+++	∝	C	∝	C	,,	C
4	0	4	2,565	+++	,,	C	,,	C	,,	C
5	0	2	..	+	,,	C	,,	C	,,	C
7	0	0	167	+	,,	C	,,	C	,,	C
8	0	0	0	36	,,	C	,,	C	,,	C
10	0	0	0	2	,,	C	,,	C	,,	C
11	0	0	0	0	,,	C	,,	C	,,	C

∝ Uncountable.

The maximal inocula sterilized in different concentrations of Su do not show a simple arithmetical relationship to one another. As an extreme example, an inoculum of 10 in 1/100,000 Su grew out readily, after the usual period of growth followed by temporary bacteriostasis, whereas an inoculum of 30×10^9 in 1/100 Su was finally sterilized. This relationship is well brought out in Table II, and examples will be seen in other tables. It is evident that the minimal effective (i.e. bactericidal) B : Su ratio rises as the Su concentration falls, the rise being particularly steep from dilutions of 1/100 to 1/500 and again from 1/10,000 to 1/100,000. It seems that in the initial growth phase, a certain " critical " bacterial density must be reached

in any one concentration of Su if the organism is to survive and ultimately proliferate.

TABLE II.—*Minimal Bactericidal Su : B Ratios (mg. of Su per 100,000 Bacteria) with Increasing Dilutions of Sulphanilamide in Nutrient Broth.*

Su dilution.	Su : B ratio.	Survival time of culture (days).
1/100	0·00003*	21
1/500	0·1	9
1/1000	0·25	9
1/2500	0·40	8
1/5000	1·0	8
1/10,000	2·5	7
1/20,000	10·0	11
1/50,000	40·0	13
1/100,000	400·0	6

* At this concentration of Su no practicable inoculum of *Br. abortus* survived as long as the control, though with added " P " factor the survival time was prolonged to 30 days.

It can be seen (Table I) that in 1/1000 Su the bacterial population reached roughly $6{\cdot}5 \times 10^6$ per ml., and then the number of viable bacteria fell rapidly to zero. Actually it was found that in 1/1000 Su a population in the region of 10^8 must be attained if the culture is to survive. In 1/10,000 Su the population must reach about 10^7 if it is to survive. A sufficient number of viable counts with different sized inocula in ascending dilutions of Su have not been made, but analysis of a mass of data based on indirect methods of assessing the number of viable bacteria strongly suggested that the " critical " bacterial density necessary for survival decreases in direct ratio with the dilution of Su. In dilutions of 1/100 Su and below it was found impossible to produce progressive growth even with an initial inoculum of 30×10^9. The survival time, however, increased progressively, even at this high Su concentration, with every large increase in the initial inoculum. At this high bacterial density, however, the factor of available food supply is a complication.

All organisms proliferating in Su solution show a preliminary active growth phase which with rapidly growing organisms, such as the streptococcus and pneumococcus, lasts for a few hours. In the case of *Br. abortus* this active growth phase varies from a few hours in a concentration of 1/100 Su in broth to two to three days in 1/10,000 Su. After this phase bacteriostasis commences, and either continues to final sterilization or after a period of hours, days or even weeks, is followed again by active growth of the culture.

The final bacterial density attained is a product of two factors : (1) the size of the initial inoculum and (2) the concentration of Su. The greater the inoculum in a particular concentration of Su, the sooner is the " critical " bacterial density reached ; the greater the concentration of Su, the more rapid is the bacteriostatic effect. The B/Su ratio rises rapidly with increasing dilutions of Su because the preliminary active growth period is prolonged ; in the weaker concentrations, Su reaches a lethal concentration in or on the organism more slowly.

RELATIONSHIP BETWEEN THE NUTRIENT MEDIUM AND THE ACTION OF SU.

A precise relationship between the bacterial density and the survival of the culture was found in media other than plain broth, such as serum, liver, broth, etc. In early experiments the wide differences in potency of Su in various nutrient media were interpreted variously. Later it became clear that the nature of the medium merely affected the growth rate according to its capacity to supply the nutritional requirements of the organism. For instance, in glucose broth, which is a relatively poor culture medium for *Br. abortus*, a very much larger inoculum was destroyed than in plain broth (see Table III). As an example, the effective Su/B ratio at a 1/5000 Su dilution was roughly a hundred times less in glucose than in plain broth. This was thought to be due to the greater sensitivity of the organism under poor nutritional conditions to Su, but it was later shown that the presence of glucose greatly retarded the momentum of the initial growth phase so that a much larger inoculum was necessary if the " critical " bacterial density was to be reached.

TABLE III.—*Comparison between Effect of Sulphanilamide on* Br. abortus *in Plain and Glucose* (2%) *Broth. Example of Apparent Growth-stimulating Effect of Sulphanilamide in Glucose Broth.*

Su dilution.	Presence of glucose.	Inoculum.	Hours.	Growth developing from explant. Days.								
			2.	1.	2.	3.	4.	6.	8.	10.	14.	18.
1/5,000	—	2×10^5	C	+++	C	C	C	C	C	C	C	C
1/5,000	+	2×10^5	C	++	+++	++	+	11	0	0	0	0
1/20,000	+	2×10^5	C	+++	+++	+	9	+	+	+	++	C
1/10,000	+	2×10^3	+++	SC	+++	+	0	0	0	0	0	0
No Su	+	2×10^3	1	1	2	4	2	0	0	0	0	0

In blood serum the minimal effective Su/B ratio for *Br. abortus* was much the same as in broth, but in defibrinated blood the ratio was considerably lower and showed wide species and individual variations. The ratio in rat blood was much higher than in human blood, whilst in human blood itself the ratio varied considerably, e.g. in 1/5000 Su an inoculum of 2×10^7 was killed in blood from a case of recovered undulant fever, compared with 2×10^5 in a sample of normal human blood and 2×10^4 in nutrient broth. When defibrinated blood was heated at 56° C. for 30 minutes prior to testing, the effective Su/B ratio rose and did not vary greatly from that of the broth control. These facts suggest that further destruction, by phagocytosis of the Su-treated organisms was responsible for the sterilization of a much larger inoculum by fresh blood than by serum or broth.

According to the " critical " bacterial density theory, the survival time of *Br. abortus* in a solution of Su in saline should depend directly upon the number of organisms inoculated, for in this case there will be little or no pullulation. In Table IV it is shown that in 1/1000 Su, an inoculum bringing the density of the culture to about the " critical " level (10^8) survived nearly as long as the control without Su, whilst an inoculum of 10^9 survived for exactly the same time.

TABLE IV.—*Action of Sulphanilamide on* Br. abortus *in Saline* (0·85%).

Inoculum per ml.	1/1000 Su. Survival time (days).	Saline alone. Survival time (days).
10^5	1	10
10^6	2	16
10^7	4	17
10^8	15	17
10^9	21	21

THE HEAT-SENSITIVITY OF SULPHANILAMIDE-TREATED BACTERIA.

It has already been mentioned (see "Methods") that a culture of *Br. abortus* showing progressive bacteriostasis in Su is very easily killed by heat. In the experiment in saline referred to in the last section, the plates used for viable counts were sterile several days before the plates inoculated from a loop explant. Examples of a similar effect in broth will be seen in Table I. The only feasible explanation was that when the explant was made into melted agar for a viable count, the exposure to a temperature of 42°–45° C for a very short period (not more than one minute) was sufficient to kill the surviving bacteria. Experiments to test this point have shown that the longer the bacteria have survived in Su the more sensitive they are to heat. The finding may be of some importance in relation to Su therapy.

When bacterial metabolism is suspended by the action of cold the effect is striking. Sulphanilamide had no bactericidal action whatsoever on *Br. abortus* at 0° C., even after three days' exposure to a 1/1000 solution in broth. Bliss and Long (1937) report a similar finding using streptococci.

These results are mentioned here, as the acceleration of Su action by heat and its inhibition by cold are facts which support the hypothesis that Su inhibits some vital enzyme reaction in the bacterial cell.

"ESCAPE" FROM THE BACTERIOSTATIC ACTION OF SULPHANILAMIDE.

As explained previously (see "Methods") it was necessary to make daily explants from cultures of *Br. abortus* in Su for long periods, and the following striking sequence of events was frequently observed.

When the size of the inoculum was about that of the maximum inoculum sterilized by the particular concentration of Su used, the culture, after being apparently sterile for periods varying from 1 to 21 days, would in sub-culture again reveal the presence of viable bacteria. At first, one or two colonies would develop from the loop explant on agar, increasing in number slowly for a few days, and finally culminating in rapid outgrowth.

Many attempts were made to grow the organisms during this "sterile" stage by diluting the bulk of the culture in a large volume of broth. Only rarely was a positive culture obtained, followed by relatively slow outgrowth : in all these instances, however, one or more colonies developed one to five days later from the routine explants on to solid agar. In these apparently sterile cultures, in which later growth begins anew, some organisms must still remain alive but

TABLE V.—*Examples of "Escape" Effect With or Without Added "P" Factor* (Br. abortus *Extract*).

Su dilution.	Inoculum per ml.	Presence of "P" factor.	Growth developing from explant. Days. 1.	2.	3.	4.	6.	8.	10.	14.	18.	22.	26.	30.
1/1,000	2×10^5	−	SC	+++	+++	+++	+	0	0	0	0	0	0	0
	2×10^5	−	SC	SC	SC	+++	+	14	1	1	1	4	+	C
	2×10^5	−	SC	+++	SC	++	+	16	1	0	0	0	+	C
	2×10^3	−	+	++	+	0	0	0	0	0	0	0	0	0
	2×10^3	+	++	+++	+++	++	0	0	2	3	+	+++	C	C
1/10,000	2×10^3	−	+	++	++	+	33	10	1	0	0	0	0	0
	2×10^3	−	++	++	++	++	++	+	0	0	30	+	C	C
	2×10^2	−	+	++	+	1	0	0	0	0	0	0	0	0
	2×10^2	+	+	++	++	++	40	0	+	++	C	C	C	C
1/20,000	2×10^3	−	+++	+++	+++	++	+	4	12	20	++	++	C	C
	2×10^2	−	+	++	++	+	9	1	0	0	0	0	0	0
	2×10^2	+*	+	+	+	0	0	0	1	4	++	C	C	C
1/50,000	2×10^2	−	3	+	18	2	0	0	0	0	0	0	0	0
	2×10^2	−	5	+	+	1	0	0	0	0	1	28	+	C
	2	−	1	5	0	0	0	0	0	0	0	0	0	0
	2	+	8	14	+	++	+	9	0	0	+	+	++	C

* Concentrated N/25 NH_4OH extract from rat liver.

be incapable of reproduction. Some change must occur in the organism or in the nutrient medium before the reproductive power is re-established.

In Table V a few examples illustrative of the " escape " effect are given. It has been observed on numerous occasions in broth, and more rarely in defibrinated blood.

The strain of *Br. abortus* growing out under these conditions was Su-resistant, but only relatively and not absolutely so. In fact, the degree of resistance was directly related to the period the culture had survived in Su and also to the concentration of Su. Examples of this relationship are given in Table VI. The development or selection of a strain growing more readily in Su occurred whenever *Br. abortus* survived in Su for more than a few days. The development of resistance is not the direct cause of the " escape " effect, but mainly incidental to it. It is later shown that this " escape " effect is probably due to the slow accumulation of " P " factor released from dead or dying organisms.

TABLE VI.—*Difference in Resistance to Sulphanilamide* (1/10,000 *in Broth of Strains of* Br. abortus *Previously Surviving in Sulphanilamide for Various Periods.*

Previous history of strain.			Inoculum per ml.				
			2.	20.	200.	2000.	20,000.
Growth in 1/1000 Su.	Presence of " P " factor.	Days surviving in 1/1000 Su.	Appearance of turbidity (days).				
" Escape " after 15 days' " sterility "	+	60	5	4	3	2	1
" Escape " after 5 days' " sterility "	0	50	8	7	6	4	3
Progressive	0	55	9	9	5	3	2
,,	+	14	∝	∝	13	12	7
,,	+	No Su 20 days in broth	∝	∝	∝	∝	12
Stock strain.	—	0	∝	∝	∝	∝	20

∝ No turbidity at 40 days.

INQUIRIES INTO THE RELATION BETWEEN BACTERIAL DENSITY AND INHIBITION OF SULPHANILAMIDE ACTION.

It appeared much more likely from the start that the bactericidal action of Su was not due to a combination with, or destruction of, some essential nutrient in the medium, for if so, the greater the bacterial density the earlier sterility would occur ; precisely the opposite occurs. Moreover, in the presence of Su there was an initial period of active growth at a progressively diminishing rate, and this continued for a period which was inversely proportional to the concentration of Su. It seemed easier to explain this growth phase by assuming that Su by diffusion into, or absorption by, the organism gradually reached a bacteriostatic concentration in the cell, than by assuming a slow combination of Su with some essential nutrient substance.

The fact, however, that the effectiveness of Su diminished as the bacterial density increased suggested that Su might be broken down or chemically modified by the action of bacteria. Numerous analyses (method of Marshall, 1937) of broth cultures in which *Br. abortus* had survived for long periods in the presence of Su have not revealed (according to the results of this colorimetric method) the slightest loss of sulphonamide. For instance, a 1/20,000 Su broth culture made with a heavy inoculum (10^8), which grew out almost as quickly as in plain broth, showed, after six weeks' incubation, no greater loss of sulphonamide than the control non-inoculated broth.

Also, a thick suspension (roughly 5 per cent. by volume) of *Br. abortus* in 1/1000 Su in saline showed no significant loss of Su after seven days' incubation. The amount in the supernatant fluid after centrifuging was reduced to an extent which could be accounted for by a slightly higher concentration in the cell than in the medium. No difference was found in this connection between a Su-sensitive and a Su-insensitive strain. When enormous quantities of bacteria with a high metabolic rate are suspended in broth there is some loss of Su. This finding has no relation to the usual conditions under which the organism grows in Su. It will be considered fully in a later publication.

The possibility still remained that some group in the Su molecule, other than the amino group responsible for the diazo reaction, was altered so that the biological activity of Su was modified. This was tested as follows: Broth cultures, with graded concentrations of Su, were seeded with an inoculum of 2×10^5, and after three weeks' incubation filtered. The filtrates were then diluted with one part of fresh broth and re-inoculated with a graded series of inocula to determine their bactericidal potency. It was found that where there was progressive growth in the original cultures the filtrate had lost some activity, but if sterilization had occurred there was no significant loss. Where loss of Su activity occurred, the filtrates killed 20–40 per cent. fewer organisms than were killed by similar Su concentrations in the controls. This loss of Su activity and the course of growth in the re-inoculated filtrates (in which the "escape" effect frequently occurred) precisely resembled results seen later, when small amounts of "P" factor were added to media containing Su. As will be seen, filtrates from *Br. abortus* cultures do, in fact, contain "P" factor in amounts which progressively increase with the age of the culture. Nevertheless the fact that a decrease in bactericidal potency of the filtrates only occurred when there was progressive growth in the culture could still have been accounted for by some chemical change in part of the Su present.

It was finally conclusively proved that most, and probably all, the Su remained intact. Nearly the whole amount (90 per cent.) of the Su in a 1/1000 solution in broth, in which *Br. abortus* had grown freely and remained viable after one month's incubation, was recovered. The crystalline Su finally obtained had the melting-point and biological activity of the pure substance.

The conclusion was then reached that under ordinary cultural conditions Su did not undergo chemical combination or destruction either in the medium or the cell, and that in all probability it acted in or on the cell by inhibiting some phase of bacterial metabolism. It seemed that progressive growth of an organism in the presence of Su might be due to the release of some factor from the organism, which, when present in a sufficient concentration, could

antagonize the bacteriostatic action of Su. The relationship of growth in Su to the " critical " bacterial density, the " escape " effect and the integrity of the Su molecule throughout the process all fitted in well with this hypothesis.

SEARCH FOR THE ANTI-SULPHANILAMIDE FACTOR IN *BR. ABORTUS* EXTRACTS.

Early experiments are only mentioned briefly if they have still some significance. The following are examples of *Br. abortus* preparations tested for possible anti-Su action :

(1) Suspension (10^{11}) in saline, incubated at 37° C. for 1 to 30 days ; heat killed. (2) Filtrates from (1). (3) Suspensions (10^{9}) in distilled water incubated at 37° C. for seven days. (4) Filtrates from (3). (5) Similar suspensions in the presence of Su (1/100–1/200). Bacteria centrifuged down and resuspended in saline ; heat killed. (6) Filtrates from pancreatin digests (pH 8·0) of similar suspension—one to seven days at 37° C. ; neutralized. (7) Broth cultures incubated for one day to six weeks ; heat killed. (8) Filtrates from (7).

Of these preparations, only (4) and (8) showed any evidence of antagonizing the action of Su. In fact, many of the long-standing bacterial suspensions in saline accentuated the action of Su, probably, as later work indicated, owing to the presence of growth-inhibiting substances : a similar inhibitory effect was seen in old broth cultures and their filtrates. At a later stage a 90 per cent. alcohol-soluble concentrate, prepared from the filtrate from a six weeks' broth culture, was found to have a very strong anti-Su action, despite the fact that the growth-inhibiting substances were not all removed by this treatment. The filtrates from short-term (one to seven days) broth cultures were sometimes feebly active, but there was always some evidence of anti-Su activity when the undiluted filtrates were used as the test medium. (The preparations were usually diluted 5–50 times in the test medium.)

Filtrates (4) from the suspensions which were simply allowed to autolyze in distilled water,though relatively feeble, were the most active of these early extracts. It was later found that methods in which this technique was used for the first extraction, using around 1·5–2·0 g. bacteria (dry weight) and about 50 ml. of H_2O, yielded very potent extracts.

DILUTE AMMONIA EXTRACTS OF *BR. ABORTUS*.

At this stage, however, Stamp (1939) published his finding of an anti-Su factor in the streptococcus, based on the observation that heat-killed streptococci were feebly inhibitory to the action of Su on like organisms.

I had previously tested a suspension of heat-killed haemolytic streptococci, using *Br. abortus* as a test organism, with negative results. The explanation of this difference probably lies in the fact that the strain of streptococcus used by Stamp is less sensitive to Su than is *Br. abortus*. The less Su-sensitive the organism, the less is the anti-Su factor required to inhibit the bacteriostatic effect of Su. In a rather similar way the activity of an extract was detected in much greater dilution than in the test method described (see " Methods "), if the experimental conditions were such that a delicate balance between

possible outgrowth and possible stasis was present; i.e. if the medium itself contained nearly, but not quite enough anti-Su factor to inhibit the Su present.

TABLE VII.—*Examples of Anti-sulphanilamide Effect of Crude* Br. abortus *Extract (AC)a on Growth of* Br. abortus *in Nutrient Broth.*

Su dilution.	Presence of "P" factor.	Inoculum per ml.	Turbidity. Days. 1.	2.	3.	4.	6.	8.	10.	14.	18.
1/1,000	+	2×10^5	0	0	+	+	++	++++	−	−	−
		6×10^4	0	0	0	0	0	+	++	+++	−
		2×10^4	0	0	0	0	0	0	0	0	0
		*2×10^5	0	0	+	+	++	++++	−	−	−
		*6×10^4	0	0	+	+	++	+++	++++	−	−
		*2×10^4	0	0	0	0	0	+	+	++++	−
1/1,000	−	2×10^5	0	0	0	0	0	0	0	0	0
		6×10^4	0	0	0	0	0	0	0	0	0
		2×10^4	0	0	0	0	0	0	0	0	0
1/10,000	+	2×10^3	0	0	0	0	++++	−	−	−	−
		200	0	0	0	0	+++	++++	−	−	−
		20	0	0	0	0	0	+++	++++	−	−
		2	0	0	0	0	0	+	+++	++++	−
1/10,000	−	2×10^3	0	0	0	0	0	0	0	0	0
		200	0	0	0	0	0	0	0	0	0
		20	0	0	0	0	0	0	0	0	0
		2	0	0	0	0	0	0	0	0	0

Su dilution.	Presence of "P" factor.	Inoculum per ml.	Growth developing from explant. Days. 1.	2.	3.	4.	6.	8.	10.	14.	18.
1/1,000	+	2×10^5	+	+++	+++	SC	C	−	−	−	−
		6×10^4	+	+++	+++	SC	+	C	−	−	−
		2×10^4	++	+++	+++	SC	6	1	2	0	0
		*2×10^5	++	SC	C	−	−	−	−	−	−
		*6×10^4	+	SC	C	−	−	−	−	−	−
		*2×10^4	20	+++	SC	+++	+++	SC	C	−	−
1/1,000	−	2×10^5	+++	++	+++	SC	+	0	0	0	0
		6×10^4	++	++	+++	+++	4	0	0	0	0
		2×10^4	++	+	+	18	0	0	0	0	0
1/10,000	+	2×10^3	++	+++	C	−	−	−	−	−	−
		200	+	++	SC	C	−	−	−	−	−
		20	21	+	++	+++	C	−	−	−	−
		2	3	11	+	++	+++	C	−	−	−
1/10,000	−	2×10^3	++	+++	++	+	25	0	0	0	0
		200	+	+	+	19	0	0	0	0	0
		20	8	17	4	0	0	0	0	0	0
		2	0	1	2	1	0	0	0	0	0

* *Br. abortus* extract prepared by extracting with N/25 NH_4OH in the presence of 1 per cent. sulphanilamide.

Stamp's method was applied to *Br. abortus*, and the first experiment (see Table VII) showed that a crude neutralized NH_4OH extract was relatively rich in anti-Su factor. This experiment is the prototype of many, though with modifications of this method much more potent extracts were later obtained. As has been indicated (see " Methods ") ammonia extraction is not the only, nor the best method of extracting the anti-Su factor, but such extracts, and concentrates from them, were mainly used in the work described here.

That the effects of the extract exactly resemble that obtained by a simple increase in the bacterial density was soon clear, i.e. (1) immediate progressive growth ; (2) rapid growth after a short period of bacteriostasis ; (3) gradual bacteriostasis and apparent sterilization followed by the " escape " effect ; or (4) final death of the culture after a longer survival time. In other words, the result of adding the extract to various concentrations of Su, which in the absence of extract killed the inoculum used, was precisely similar to that produced by simple increases in the initial inoculum.

THE RELATION BETWEEN THE GROWTH-STIMULATING FACTOR (" P " FACTOR) AND THE ANTI-SULPHANILAMIDE FACTOR IN *BR. ABORTUS* EXTRACTS.

Might not the anti-Su action be simply due to a stimulation of bacterial growth ? Stamp did not test this possibility, but tests on *Br. abortus* in nutrient broth (see Tables VIII and IX) showed that growth was greatly stimulated by even a crude NH_4OH extract of *Br. abortus*. Using a concentrated extract, the reproduction rate in 1/2500 Su was very much greater even than that in plain, untreated broth.

TABLE VIII.—*Stimulation of Growth of* Br. abortus *by "P" Factor* (Br. abortus *and* Ps. pyocyaneus *Extracts* $(AE)a + (AE)b$).

Inoculum per ml.	Number of bacteria per ml. after 24 hours.				
	Broth alone.	Broth + *Br. abortus* extract.	1/10,000 Su. in broth.	1/10,000 Su + *Br. abortus* extract.	1/10,000 Su + *Pyocyaneus* extract.
2	750	4,450	—	—	—
20	2,350	50,850	1,350(*a*)	5,150(*b*)	2,500(*c*)
200	54,000	∝	20,550(*d*)	112,500(*e*)	63,000(*f*)

Cultures (*b*) and (*e*) showed progressive growth, turbidity first appearing at the 9th and 7th days. Cultures (*a*), (*c*), (*d*) and (*f*) showed diminishing numbers of viable bacteria and were sterile at the 4th, 5th 6th and 13th days.

It seemed very probable that the anti-Su effect of " P " factor was purely the result of its growth-stimulating action, and that, though highly significant in itself, it had no fundamental relationship to the mode of action of sulphanilamide. The following evidence, however, strongly suggests that " P " factor antagonizes the action of Su in some direct manner.

If the anti-Su action of " P " factor was an indirect one, if might inhibit the bacteriostatic action of an antiseptic substance chemically differing from

sulphanilamide. Its action on thionin (1 : 50,000), which in this concentration is bacteriostatic but not bactericidal to *Br. abortus*, was tested, but in its presence *Br. abortus* extract (AC)a did not stimulate growth. Actually thionin appeared to inhibit growth rather more in the presence of " P " factor than in its absence. This finding suggested that " P " factor might inhibit the action of Su in some direct manner, and so the following more crucial experiment was tried: To one series of Su dilutions in broth, *Br. abortus* extract (AE)c was added at the time when, as will be seen from Table X, the cultures were apparently sterile. The inhibitory effect of an equivalent amount of NH_4Cl in the controls should be noted. Under these conditions there was no question of a pure growth-stimulating effect during the initial growth phase, before Su had produced its full bacteriostatic effect. Nevertheless, after a period increasing directly as the size of the initial inoculum decreased, turbidity developed in the cultures and rapid outgrowth occurred.

TABLE IX.—*Stimulation of Growth of* Br. abortus *by* Br. abortus *Extract (AE)a + (AE)b in Plain and Glucose* (1%) *Broth.*

Type of broth.	Inoculum per ml.	Presence of " P " factor.	Growth developing from loop explant. Days. 1.	2.	3.	4.
Plain	2	+	2	+++	C	C
,,		−	1	+	SC	C
Glucose		+	0	+	SC	C
,,		−	0	13	+++	C
Plain	20	+	15	SC	C	C
,,		−	5	+++	C	C
Glucose		+	7	+++	C	C
,,		−	1	+	C	C
Plain	200	+	+++	C	C	C
,,		−	+	C	C	C
Glucose		+	+	C	C	C
,,		−	15	++	C	C

This experiment, which has been repeated with similar results in various Su concentrations, seems to prove that " P " factor has a direct antagonistic action on sulphanilamide and is thus identical with the anti-Su factor. Moreover, though complete data have not yet been obtained, it appears that there is a direct quantitative relationship between the concentration of Su and that of " P " factor required to inhibit it.

This stimulating effect of " P " factor on *Br. abortus* in the " non-reproductive " phase was also shown in the following way: Explants on to agar from *Br. abortus* cultures in Su solutions, made on the first and second day of apparent " sterility," were observed for three weeks to make sure that no delayed outgrowth was occurring. The plates were then smeared with a few drops of concentrated *Br. abortus* extract, and in many cases colonies of *Br. abortus* then developed, often quite rapidly. " P " factor had stimulated reproduction in an organism which had lost that capacity. In those cases

TABLE X.—*The Anti-sulphanilamide Action of "P" Factor. Comparison between Effect of Adding* Br. abortus *Extract (AE)b at the same time as, and Five Days after Adding the* Br. abortus *Inoculum (Nutrient Broth—*1/1000 *Su).*

Inoculum per ml.	Turbidity. Days. 1.	2.	3.	4.	5.	6.	8.	10.	14.	18.	22.	26.	30.
6×10^{4}*	0	0	+	++	+++	+++	++++	−	−	−	−	−	−
2×10^{4}*	0	0	0	+	++	+++	++++	−	−	−	−	−	−
2×10^{3}*	0	0	0	0	0	+	+++	++++	−	−	−	−	−
200*	0	0	0	0	0	0	0	0	0	0	0	0	++++
20*	0	0	0	0	0	0	0	0	0	0	0	0	0
6×10^{4}	0	0	0	0	0*	+	++	++	++++	−	−	−	−
2×10^{4}	0	0	0	0	0*	0	+	+	+++	++++	−	−	−
2×10^{3}	0	0	0	0	0*	0	0	0	0	0	+	++++	−
200	0	0	0	0	0*	0	0	0	0	0	0	0	0
Controls — 1/1,000 Su + M/20 NH_4Cl (*no* Br. abortus *extract*).													
2×10^{5}	0	0	0	0	0	0	0	0	0	0	0	0	0
6×10^{4}	0	0	0	0	0	0	0	0	0	0	0	0	0

Inoculum per ml.	Growth developing from explant. Days. 1.	2.	3.	4.	5.	6.	8.	10.	14.	18.	22.	26.	30.
6×10^{4}*	++	+++	SC	SC	SC	SC	C	−	−	−	−	−	−
2×10^{4}*	++	+++	+++	C	−	−	−	−	−	−	−	−	−
2×10^{3}*	++	+++	+++	+	18	0	2	3	++	C	−	−	−
200*	25	12	0	0	0	0	0	0	0	42	1	+	C
20*	4	1	0	0	0	0	0	0	0	0	0	0	0
6×10^{4}	+	+	0	0	0*	+++	C	−	−	−	−	−	−
2×10^{4}	+	10	0	0	0*	+++	+++	SC	C	−	−	−	−
2×10^{3}	+	0	0	0	0*	+	+	20	30	++	C	−	−
200	30	20	0	0	0*	0	0	0	0	0	0	0	0
Controls — 1/1,000 Su + M/20 NH_4Cl (*no* Br. abortus *extract*).													
2×10^{5}	10	0	0	0	0	0	0	0	0	0	0	0	0
6×10^{4}	5	0	0	0	0	0	0	0	0	0	0	0	0

* "P" factor added.

where no growth occurred, it is presumed that the original culture was truly sterile, the bacteria having passed the quiescent non-reproductive stage and died.

No evidence has been obtained of any chemical combination of " P " factor with Su, and it would appear probable that " P " factor specifically stimulates that chemical reaction in the bacterial cell which Su inhibits.

THE LACK OF SPECIES SPECIFICITY IN THE ACTION OF " P " FACTOR.

If " P " factor specifically inhibited the action of Su, the *Br. abortus* extracts would be expected to inhibit the action of Su on all Su-sensitive organisms. This proved to be so with all those tested, but there is only space for a few details.

The " P " factor extracts from *Br. abortus* were found to inhibit the action of Su on two strains of *haemolytic streptococci*, *Bact. coli*, a meningococcus and a pneumococcus, and the effect was just as definite on organisms relatively insensitive to Su, such as *Ps. pyocyaneus*, various diphtheroids and several others. In broth, for instance, a Su concentration of 1/500 was bactericidal to an inoculum of *Ps. pyocyaneus* not exceeding 20 organisms : in the presence of added " P " factor an inoculum of two grew out in 24 hours. It may be mentioned here that *Ps. pyocyaneus*, in the presence of " P " factor and Su, has also been observed to grow out in the " escape " manner.

Not only does " P " factor show no species specificity in its anti-Su action, but none in its growth-promoting action so far as has been tested. Complete quantitative data cannot yet be given, but relatively crude tests (see " Methods ") revealed a more or less powerful growth-stimulating action under ordinary culture conditions on ten different strains of streptococci (haemolytic and non-haemolytic) and on the following organisms : *Br. paramelitensis*, *Ps. pyocyaneus*, *Bact. coli*, *Bact. typhosus*, *B. anthracis*, *B. proteus*, *B. dysenteriae* (Flexner Y), *Staph. aureus* and a meningococcus.

This growth-stimulating effect is particularly well shown under poor nutritional conditions. The data in Table IX illustrate this effect, for in this case the growth-rate of *Br. abortus* in glucose broth (a poor medium) in the presence of a low concentration of " P " factor reached the level seen in plain broth. Cultures of haemolytic streptococci and meningococci did not grow sufficiently in plain broth to produce any turbidity, whereas when " P " factor (10 per cent. of crude extract) was added, the cultures were distinctly turbid within 24 hours, though not so markedly as cultures in serum broth alone.

This non-specific growth-stimulation is often astonishingly great, and " P " factor preparations and media containing it are easily contaminated with a striking variety of bacteria and moulds.

It has not yet been decided whether, the more sensitive an organism is to Su, the more it responds to the growth-stimulating effect of added " P " factor. At any rate, Su-insensitive organisms (e.g. two Aronson strains of haemolytic streptococci) certainly showed growth-stimulation by " P " factor.

THE LACK OF SPECIES SPECIFICITY IN THE SOURCE OF "P" FACTOR.

Extracts have been obtained by like methods from a number of bacterial species, which differ in no essential manner in their anti-Su and growth-stimulating action from those obtained from *Br. abortus*. The scope of this work has been greatly enlarged, but the details must be held over.

An example showing the presence of "P" factor in a crude ammonia extract of *Ps. pyocyaneus* is given in Table VIII, but it is not meant to show comparable potencies, for the pyocyaneus extract was prepared from a much smaller weight of organisms than was the *Br. abortus* extract. Extracts containing "P" factor have been obtained from *B. proteus*, *Bact. coli*, *Staph. aureus*, *Br. paramelitensis*, and a Su-insensitive strain of *Staph. albus*, but not as yet from tubercle bacilli. There is now evidence that it is produced by a wide variety of bacteria, possibly by all.

As yet there is no evidence that the more insensitive the organism is to Su, the greater the amount of "P" factor it yields. For instance, a very Su-resistant strain of *Br. abortus* yielded potent extracts, but not as potent as comparable extracts made from the Su-sensitive strain, and no organism up-to-date has yielded more if as much as the latter organism. The extract from the Su-insensitive strain of *Staph. albus* was relatively weak, and it is possible that much of the "P" factor was lost from the Su-insensitive bacteria by the preliminary washing in water* (see "Methods"), for there is evidence that "P" factor is released from the Su-insensitive organism much more readily than from *Br. abortus*.*

It is suggested that the sensitivity of an organism to the action of Su depends on the amount of "P" factor which it liberates into the surrounding medium in a unit of time. If the concentration of "P" factor rises to a level sufficient to neutralize the bacteriostatic action of Su at a time when some organisms are still viable, then the culture will survive.

THE MODE OF PRODUCTION OF "P" FACTOR.

The sequence of events in the "escape" effect suggested that "P" factor was formed or released during autolysis of the organism. This effect occurs when practically all the organisms have been killed. If "P" factor were released from the living organism it would be necessary to assume that during the "sterile" stage a few living cells, with a very low metabolic rate, could release enough "P" factor into the medium to neutralize the Su present, whereas the concentration inside the cell was insufficient to do this. This seems unlikely.

Other findings support the hypothesis that it is released during autolysis of the dead or dying cells. Although "P" factor will resist boiling in N/1 NaOH, bacterial extracts made by incubation with dilute NH_4OH diminished in potency the more the concentration of NH_4OH exceeded N/25. It is possible that the higher concentrations of NH_4OH inhibited autolysis. Moreover, if the organisms, killed by four days' incubation in N/25 NH_4OH, were

* Proof of these suggestions has now been obtained.

resuspended in distilled water, still greater amounts of " P " factor were released, and even then a further yield was obtained by boiling the organisms in water.

The filtrates from a thick suspension of *Br. abortus* (which had a thorough preliminary washing) in distilled water contained, after nine days' incubation, much more " P " factor and much less solid material than the preparation made by extraction for two days with dilute ammonia, and the amount of " P " factor in the autolysate increased progressively during the period of incubation. Similarly, the longer the period (up to six weeks) of incubation of broth cultures, the greater was the amount of " P " factor extractable from them. On the other hand, suspensions of *Br. abortus* in saline did not yield very active extracts, possibly because under these conditions most of the organisms remain viable.

It would seem that " P " factor is released and possibly produced (though this is more uncertain) during autolysis of the bacterial cell.

THE RELEASE OF " P " FACTOR FROM THE BACTERIAL CELL.

A few workers, particularly Finklestone-Sayliss, Paine and Patrick (1937), have observed that streptococci in the presence of Su proliferate more rapidly during the initial growth phase than control cultures.

In many early experiments with *Br. abortus* a rather similar effect was observed, but owing to its erratic occurrence, the conditions favouring its appearance were difficult to analyse. It seemed, however, that it was particularly under poor nutritional conditions that this accelerated growth took place. Instances were seen where in glucose broth inoculated with organisms from an old culture, outgrowth failed to occur, whereas in glucose broth containing Su, there was progressive growth for some time (see Table III), followed by death of the culture.

This surprising event was later observed under different conditions. On several occasions a small inoculum of a Su-sensitive streptococcus in plain broth, containing added " P " factor, grew much better in the presence of Su than in its absence, though in both cases the culture perished completely in 48 hours ; as usually happens when stimulation by " P " factor occurs in a deficient medium. Under similar conditions, the meningococcus and a slow-growing diphtheroid have behaved in the same way. In serum broth, in which the streptococcus survives much longer, such an accelerating effect was not detected ; i.e. stimulation of growth by " P " factor was greater in the absence of Su. This growth-stimulating effect of Su was again haphazard in appearance, and at present it can only be stated that a deficient medium and a relatively high dilution of Su seem to favour this effect.

These observations are introduced here because they may have a bearing on the mode of release of " P " factor from the organism. It is suggested that Su brings about in some way a quicker release of " P " factor from the organism into the medium. A point in favout of this is that a crude NH_4OH extract made in the presence of 1 per cent. Su from one half of a suspension of *Br. abortus* in water, had a greater anti-Su potency than an extract prepared in a similar way, but without Su, from the other half (see Table VII).

Two explanations of the temporary growth stimulation by Su suggest themselves. By an acceleration of the death-rate of bacteria in a deficient medium Su might cause a temporary increase in " P " factor production, or by increasing the permeability of the cell membrane it might produce a more rapid release of " P " factor. It has been indicated that the wide variations in the Su-sensitivity of bacteria are better explained by variations in the rates of diffusion of "P" factor from the cell rather than by differences in the amounts produced by them. The variations in sensitivity may, of course, be due to variations in the rate at which Su diffuses into or is absorbed by the organism, but the chemical evidence (v. s.), though not conclusive, does not support this view.

The fact that more " P " factor is obtained from *Br. abortus* after two days' extraction with N/25 NH_4OH than after extraction for the same period with H_2O may be due to saponification of the fats in the cell envelope, which allows of the more rapid diffusion of the water-soluble " P " factor through it.

Telling and Oliver (1938), McIntosh and Whitby (1939) and others have shown that the bacterial capsule may disappear or show degenerative changes in the presence of Su. It is possible that such a change may increase the permeability of the cell envelope to "P" factor, and the temporary acceleration of growth observed under certain conditions might thus be produced.

The possibility that the differences found in the activity of Su compounds may be due to their effect on the cell envelope, with consequent differences in the amount of " P " factor liberated, should be considered.

SEARCH FOR " P " FACTOR IN OTHER ORGANIC MATERIAL.

For obvious reasons it was necessary to find whether extracts containing " P " factor could be prepared from organic sources other than bacteria. Various kinds of animal and vegetable material known to be rich in growth-factors were extracted (see " Methods "). The chemical details and their implications must be held over, but it can be stated that no evidence of an anti-Su factor was found in concentrated extracts from the following substances: yeast (three different sources), Marmite, meat extract (Lemco) and onions. Casein digests were also inactive. Most of these, by inhibiting bacterial growth, increased the Su effect. The negative results obtained with a variety of yeast extracts almost exclude any substance belonging to the Vitamin B group as being responsible for the anti-Su effect.

Five brands of peptone showed no " P " factor activity when added to broth at a 1 per cent. level.

Two or four highly concentrated liver (rat and beef) extracts showed very weak potency (see Table V). This does not prove that liver contains " P " factor, for from the earlier considerations it will be realized that any substance which has powerful growth-stimulating properties might show an anti-Su effect. Liver extracts might well contain such a substance, e.g. pantothenic acid. " P " factor cannot be pantothenic acid, however, for it withstands boiling in N/1 NaOH, whilst pantothenic acid is destroyed by this treatment. In view of the weak potency of the concentrated liver extracts as compared with

the crudest bacterial extracts, it is possible that in the two active extracts some mild degree of bacterial contamination during the early stages of their preparation was responsible for the presence of traces of " P " factor.

One of three concentrated extracts made from rat sarcoma (dibenzanthracene) tissue also showed feeble activity. The tumour tissue from which the active extract was made was contaminated with *B. proteus* and this was probably the source of the trace of " P " factor present.

No evidence of " P " factor activity was found in a variety of pure chemicals now known to be accessory growth-factors for bacteria. These factors are, of course, mainly " vitamin-like " in their action ; bacteria may not grow in their absence, but they do not, as " P " factor does, profoundly stimulate bacterial proliferation in an already satisfactory nutrient medium. The following substances were tested on *Br. abortus* for anti-Su potency, all with completely negative results : thiamine (B_1), nicotinic acid (and combinations of the two), β alanine, β indol acetic acid (auxin B), pimelic acid, glutamine, uracil cysteine and inositol. A preparation of crude " biotin " prepared from egg-yolk by the method of Kogl (1936), a powerful growth-stimulating substance for certain yeasts and a few types of bacteria, was also inactive. It is interesting that thiamine plus nicotinic acid was inactive, for Kerby (1939) reported that this combination enhanced the growth of *Br. abortus* on Bacto Tryptose Agar. Such stimulation was evidently insufficient to achieve the " critical " bacterial density necessary (with the experimental conditions used) to exert an anti-Su effect.

All the evidence, so far, suggests that bacteria, if not the only source, are at any rate by far the richest source of " P " factor.

CHEMICAL PROPERTIES OF " P " FACTOR.

Since further purification of " P " factor is being gradually achieved it is proposed to deal more fully with its chemical properties in a later paper. Growth-stimulation and Su-inhibition have been observed in media containing less than 1 in 10^6 parts (dry wt.) of an extract of *Br. abortus*, and there is little doubt that the pure substance must be active in very much higher dilutions.

All the active extracts were very stable, resisting repeated boiling. Extracts contaminated with luxurious moulds were still active after filtration and further boiling, and a very active extract was obtained from a *Br. abortus* broth filtrate which had stood 18 months at laboratory temperature. Even boiling for one hour in N/1 NaOH or N/1 H_2SO_4 produced little or no destruction of " P " factor. It is not adsorbed by Fuller's Earth and is probably, as Stamp (1939) suggested, in the case of his streptococcal factor, a substance of low molecular weight.

DISCUSSION.

It has been shown that *Br. abortus* survives in Su-containing media if, during the initial period of active proliferation, the bacterial density reaches or passes a " critical " level. The " critical " bacterial density increases progressively with the Su concentration. Moreover the initial period of growth diminishes rapidly as the concentration of Su rises, probably because a bacteriostatic

concentration of Su in the bacterial substance is more quickly attained. It follows, as has been experimentally demonstrated, that the size of the inoculum killed is not arithmetically related to the amount of Su present, but the ratio, Su : bacteria killed, increases rapidly as the amount of Su diminishes.

It was demonstrated that the course of growth of a bacterial inoculum which grew out in the presence of Su was exactly similar to that of a smaller inoculum which, though normally killed, grew out when a bacterial extract (" P " factor) was present. This suggested that the same factor is released into the medium during bacterial growth and that it reaches a concentration, at the " critical " bacterial density for the particular species, sufficient to completely inhibit the bacteriostatic action of Su. Strong evidence that this does actually happen, is that extracts, extremely rich in " P " factor, were obtained from filtrates of old broth cultures of *Br. abortus*.

It has been shown that a factor which antagonizes the bacteriostatic action of Su can be obtained from many and possibly all bacteria. This factor (" P " factor) has a powerful growth-stimulating action on bacteria which is not limited to the organism from which it was obtained. It appears to be released from the cell during autolysis, though whether it is produced by the living cell or by the action of autolytic enzymes in the dead or dying cell is not yet known. It is not an accessory growth-factor in the usual meaning of that term, for it strongly stimulates bacterial growth when the nutritional conditions are adequate for normal growth. It seems likely that " P " factor stimulates the reproductive process in the bacterial cell in some direct manner, rather in the same way that products of tissue necrosis (" necro-hormones ") have been supposed to stimulate the proliferation of tissue cells. Its effect on bacteria growing in a poor medium is significant in this connection, for on adding " P " factor the bacteria proliferate rapidly, but the culture quickly dies out, presumably because essential food factors are soon exhausted.

Not only does " P " factor stimulate cell-proliferation, but it overcomes the proliferation inhibiting action of Su, and there is probably a direct quantitative relation between the effect of the two substances. It has also been shown that " P " factor specifically, and not merely indirectly as a result of its growth-promoting powers, antagonizes the action of Su. No evidence has been obtained that " P " factor chemically combines with Su, and like Su, it seems to remain intact during the reaction. The conclusion seems warranted that " P " factor catalyses and Su inhibits some fundamental chemical reaction which is intimately concerned with bacterial reproduction.

The fact that mild heat accelerates and cold retards the bactericidal action of Su is strong evidence in favour of the view that the reaction which Su inhibits is enzymic in nature. " P " factor, as Stamp (1939) suggested, may act as a co-enzyme in this reaction. The evidence presented suggests that Su diffuses into, or is absorbed or adsorbed by the organism, and since it does not appear to act in the medium, the assumption seems justified that it acts within or on the surface of the cell. " P " factor also appears to act in or on the cell, for it was shown to stimulate the growth of *Br. abortus* in the non-reproductive phase induced by Su, after the organism had been removed from a medium containing Su. In this dormant phase it is suggested that the organism remains alive, but not capable of reproduction, because there is a delicate balance

between the Su and " P " factor concentrations in the cell. When this balance exists in a culture medium " P " factor may gradually accumulate in the medium owing to autolysis of the killed cells, and with increasing stimulation reproduction recommences and the " escape " effect occurs.

It is generally considered that Su is bacteriostatic to relatively few bacteria, which are mainly of high pathogenicity. It tends to be forgotten, however, that this is only a relative truth and the sensitivity to Su only varies, though very widely, in degree. Not one of the many types of organisms I have tested was completely insensitive to very high concentrations (up to 1/100) of Su. For instance, a very small inoculum of the almost insensitive *B. subtilis* was killed by a high concentration, whilst a larger inoculum showed transient bacteriostasis. It has been shown that these wide differences in sensitivity to Su are probably not due to species variations in the amount of " P " factor produced. It is therefore suggested that the rate of release of " P " factor from the autolysing bacterial cell may vary greatly, and this hypothesis is being tested experimentally.*

No evidence was obtained that " P " factor was present in any large quantity, if at all, in animal and vegetable tissues, which suggests that " P " factor is not concerned, or not of fundamental importance in the metabolism of more specialized cells. If so, the surprising fact that Su destroys bacteria in the body and leaves the tissue cells unharmed, except with massive dosage, has a rational explanation. If, as seems probable, Su has a bacteriostatic action, and " P " factor stimulates growth in some degree, in many or all bacteria, it would seem that the enzyme reaction involved is a fundamental one in bacterial metabolism.

Work is in progress on the effect of " P " factor on bacterial growth in the blood and in the body generally. Growth is usually stimulated greatly in defibrinated blood. Immunity factors appear to complicate the effect, however, and more investigation is necessary before the findings are published.

Studies of the effect of " P " factor on bacterial metabolism should throw light on the chemical reaction which Su inhibits.

Preliminary trials are being made with " P " factor in various bacteriological techniques. The uses of a substance which so powerfully stimulates bacterial reproduction may prove to be extensive.

SUMMARY.

It is suggested on the basis of numerous experiments, mainly with *Br. abortus*, that whether or not a bacterial culture survives in the presence of sulphanilamide depends on three factors :

1. The rate of proliferation in the initial growth phase. If the bacterial density in a fluid medium reaches a certain level (" critical " bacterial density) an amount of some anti-sulphanilamide factor, sufficient to prevent any further bactericidal action by the sulphanilamide present, accumulates in the medium. The amount of this factor progressively increases, and at a certain concentration bacterial reproduction is resumed.

* Recent findings give strong support to this hypothesis.

2. The rate at which sulphanilamide reaches a bacteriostatic concentration in the cell. This depends mainly on the amount of sulphanilamide in the medium, and possibly to some extent on the nature of the bacterial envelope.

3. The permeability of the cell membrane to the anti-sulphanilamide factor. This factor is released and possibly formed during autolysis of the dead or dying cell, and diffuses out into the surrounding medium. The rate at which it is released may be determined by the chemical nature of the capsule and/or cell membrane peculiar to the bacterial species or strain.

Bacterial extracts containing this anti-sulphanilamide factor powerfully stimulate the reproductive rate of many bacteria, and the factor is therefore termed provisionally " P " factor (proliferation or pullulation factor).

" P " factor appears to be identical with the anti-sulphanilamide factor, and it is suggested that it stimulates the same enzyme reaction in the bacterial cell which sulphanilamide inhibits. This reaction is probably a fundamental one in the metabolism of most or all bacteria, but not in the metabolism of other living cells.

" P " factor is not species specific either in its source or in its action.

It is very probable that the sensitivity to sulphanilamide of any bacterial species or strain is conditioned by the rate at which the autolysing organism liberates " P " factor.

Under certain conditions a balance may exist between " P " factor and sulphanilamide, and the organism is then incapable of reproduction. The living nature of the organism is only revealed when the concentration of " P " factor increases.

A sulphanilamide-resistant strain of *Br. abortus* appears when the organism survives for some time in the presence of sulphanilamide. The resistance of the strain increases the longer it survives in sulphanilamide. Resistant strains do not develop in the presence of " P " factor alone.

An explanation is suggested for the growth-stimulating action of sulphanilamide, which occurs under certain conditions.

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THE AUGMENTATION OF ACTION OF ANTHRACITE AND OTHER DUSTS BY DEAD TUBERCLE BACILLI.

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Kettle (1934) showed that an inert dust, kaolin, when injected intratracheally in rabbits along with dead tubercle bacilli, gave rise to a type of lesion which was much larger and more like a silicotic lesion than that caused by dead bacilli alone or kaolin alone. I was able to show (Cummins, 1937) that sericite, injected along with an emulsion of dead tubercle bacilli into the subcutaneous tissue of the ears of rabbits, gave rise to such a massive swelling that it tended to undergo a sterile necrosis and to discharge its contents after about six weeks, Neither quartz, with or without dead tubercle bacilli, nor sericite alone led to a discharging lesion, although all continued as hard swellings until the end of the experiment. The intratracheal injection of quartz into the lungs was greatly amplified in its effects by the intravenous inoculation after six weeks of an emulsion of dead tubercle bacilli. Compared with the lesion produced by quartz alone, this lesion was much larger and contained more giant-cells.

In view of the important bearing of these findings upon the present state of the question of nodular fibrosis in workers in the coal measures of South Wales, I decided to continue with the experiments, extending them to include some of the mixed dusts of the anthracite coal mines and the slate quarries as well as anthracite dust itself.

EXPERIMENTS.

The experiments were limited to small numbers of animals owing to the restricted space available and were, therefore, carried out over several years. In all cases the composition of the inoculum was based on that of Fallon and Banting (1935), 0·2 g. of the appropriate dust made up to 1 c.c. with sterile water or saline. To this mixture, when desired, a drop of a thick emulsion of dead tubercle bacilli was added. The whole was introduced directly into the

trachea of the rabbit, exposed for the purpose by dissection under urethane. The dusts used were " Penrhyn slate quarry dust, ' Bethesda ' finest grade—300 mesh," presented by W. R. Jones, and " Ammanford sandstone dust, ' drilling dust ' under 20μ," presented by R. Lessing. In both cases the animals injected with the combination of dust and dead human tubercle bacilli showed a much more extensive and a rather different reaction from the animals receiving the dust alone.

The same technique was followed with a pure anthracite dust, ground below 25μ, presented by R. Lessing. In this experiment, and in an experiment with quartz, a rabbit was also injected with a drop of 1/25 old tuberculin, to see whether this preparation had any adjuvant effect. While the addition of a drop of emulsion of dead tubercle bacilli led to a marked augmentation of the lesions both of anthracite and of quartz, the tuberculin had no such effect, the resulting deposits being no greater than those produced by an emulsion of the dust alone.

Only one rabbit of each group received dust together with dead tubercle emulsion but, with Penrhyn slate dust, Ammanford stone dust, anthracite and quartz, the result was markedly greater and more cellular than that produced by the same dust alone.

HISTOLOGY.

The rabbits were killed and examined after four to six months and sections were prepared from the affected lobes. Figs. 1 to 4 show sections from the lungs of rabbits which were injected with Ammanford sandstone dust and anthracite dust, with and without the addition of dead tubercle bacilli. It will be observed that the lesions produced by the dusts alone are small and discrete, hardly to be noticed without the aid of " crossed prisms " in the case of the Ammanford dust (Fig. 1) but clearly visible with the anthracite (Fig. 3). On the other hand, the lesions produced by the dusts combined with dead tubercle bacilli are large and show numbers of giant-cells within which lie masses of dust (Figs. 2 and 4). For the demonstration of the Ammanford sandstone dust and the Penrhyn slate quarry dust, which consist of sericite, quartz and other birefringent minerals, " crossed prisms " are needed. Anthracite, however, when sufficiently magnified, can be plainly seen with ordinary transmitted light inside the giant-cells of the lesion.

The micro-photographs are designed to show the crude lesions, as seen in a magnification of about 8 diameters, this comparison being sufficiently striking to show the marked amplification when dead tubercle bacilli are added. It is hoped, when further experiments have been carried out, to illustrate these with micro-photographs of higher magnifications.

DISCUSSION.

Are these lesions, so much more extensive than the lesions of dust alone, to be regarded merely as tuberculous lesions ? The fact that the giant-cells invariably contain dust particles and that there is practically no dust outside the lesions negatives this idea. No tubercle bacilli have been encountered in the lesions though carefully sought for. Kettle considered the lesions

produced by kaolin and dead tubercle bacilli as different from purely tuberculous lesions. Rabbits inoculated even with living human tubercle bacilli, apart from those that die early from allergic tuberculous pneumonia, tend to get rid of their tuberculous lesions and to be entirely free from them after two or three months (Cummins and Weatherall, 1932).

These amplified lesions would appear to be due to the action of dust and tubercle bacilli acting together, the one completing the effects of the other, so that the result cannot be attributed either to the dust alone or to bacilli alone but to a combination of the two.

SUMMARY.

Ammanford sandstone dust, Penrhyn slate quarry dust, quartz and anthracite dust all produce much larger and more cellular lesions when introduced intra-tracheally with dead tubercle bacilli than when introduced alone.

My thanks are due to Dr. W. R. Jones and Dr. R. Lessing for the dusts, and to Prof. J. B. Duguid and Mr. G. R. Armstrong, of the Pathology Department, Welsh National School of Medicine, for the micro-photographs.

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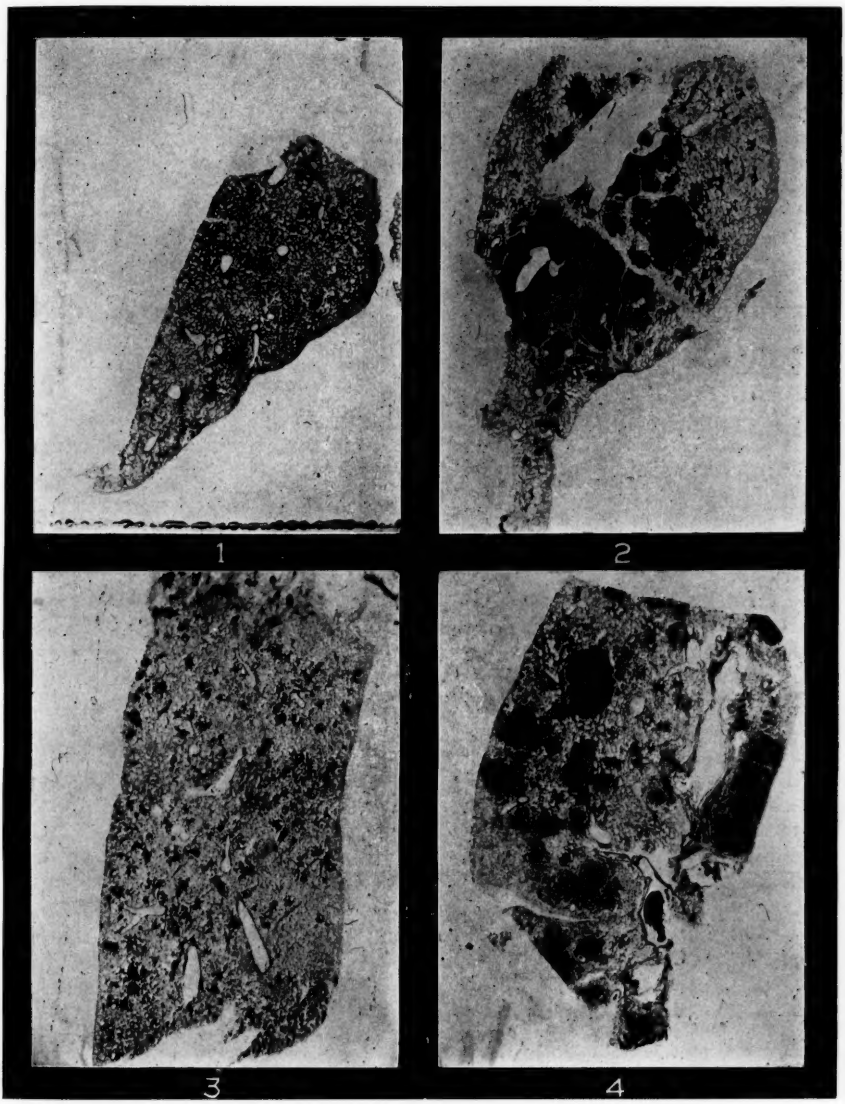

Lesions obtained in the lungs of rabbits after intratracheal injection.

Fig. 1.—Ammanford sandstone dust alone.

Fig. 2.—Ammanford sandstone dust + dead tubercle bacilli.

Fig. 3.—Anthracite dust alone.

Fig. 4.—Anthracite dust + dead tubercle bacilli.

Cummins.

UNIV. OF MICH.